

# Factors Influencing Growth and Water Quality in Experimental Abalone Culture Systems

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## **Declaration**

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## Abstract

Abalone culture is becoming increasingly important as over-exploitation and poaching worldwide are depleting wild stocks, yet demand for abalone products continues to increase. Although techniques for nursery production of juveniles and grow-out to market size are well established, optimisation of growth rates and maintaining stock health requires a better understanding of factors influencing growth, water quality and health status during the grow-out phase.

Previous studies on abalone at the University of Tasmania have investigated a range of husbandry, nutritional and water quality issues. The research covered in the current study used a range of experimental scale grow-out systems, in conjunction with a bioassay system, to investigate a number of factors previously identified as potentially affecting growth and water quality in commercial grow-out systems for greenlip (*Haliotis laevis*) and blacklip (*H. rubra*) abalone.

Previous work has investigated the effect of environmental ammonia and low dissolved oxygen levels on abalone growth as separate exposures (Harris 1999). For the present study, this work was expanded to investigate the effect on growth of low dissolved oxygen levels combined with elevated ammonia levels as both chronic exposure and as intermittent, 8-hour exposures in two separate trials. Periodic exposure to elevated environmental ammonia and 60% DO for 8 hours at intervals ranging from weekly to once in 6 weeks had no significant effect on growth for either blacklip or greenlip abalone. However, chronic exposure to similar conditions for up to 67 days resulted in significant growth reductions for both species. Disruptions in serum chemistry and ion regulation were also detected following exposure to both chronic and acute elevations in environmental ammonia levels.

Increasing the stocking density from 120 abalone/220 L tank to 360 abalone/220 L tank significantly reduced growth in terms of length, which at medium and high density was partially alleviated by increasing the level of refuge provision from 0 to 1 per 30 abalone.

Monitoring whole-tank metabolism in experimental tanks stocked at commercial rates (20 kg.m<sup>-3</sup>) over several days showed significant changes in dissolved oxygen

levels, pH and total ammonia over short (24 hour) and longer (5 day) time periods. Diurnal variations in water quality resulted from nocturnal foraging activity and post-prandial metabolism. Longer term effects were also detected as organic wastes accumulated in the tank. An earlier assessment of the pore-water quality within the organic wastes (Roden 1997) had shown that this environment was acidic, oxygen depleted and ammonia enriched. However, this potentially deleterious microenvironment had little impact on the bulk water as long as water exchange was maintained. If normal water exchange was interrupted, rapid declines in water quality occurred due to a combination of abalone and microbial metabolism in the accumulated organic wastes.

Oxygen budgets developed using community respirometry clearly demonstrated that abalone metabolism was the dominant factor influencing water quality in abalone systems. The abalone consumed 65-75% of the oxygen in the influent water at the end of the cleaning cycle, with 25-35% being consumed by accumulated organic wastes.

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**Dedicated to the Loving Memory of my Mother**

**Dawn Elizabeth Hindrum**

### **List of Abbreviations**

TA – Total Ammonia

UIA – Unionised Ammonia

DO – Dissolved Oxygen

BWQ – Bulk Water Quality

DBL – Diffusive Boundary Layer

SSA – Submerged Surface Area

EFA – Effective Foraging Area

SGR-L – Specific Growth Rate (length)

SGR-W – Specific Growth Rate (weight)

Stacking – attachment of one abalone to the shell of another

## Table of Contents

COPYRIGHT DECLARATION .....	II
ABSTRACT.....	III
ACKNOWLEDGEMENTS .....	III
LIST OF ABBREVIATIONS .....	I
<b>CHAPTER 1 - General Introduction.....</b>	<b>1</b>
1.1 MODERN INTENSIVE AQUACULTURE, GROWTH RATES AND WATER QUALITY .....	2
1.2 BIOTIC AND ABIOTIC FACTOR INFLUENCING WATER QUALITY .....	6
1.2.1 Influence of Bulk Water Exchange.....	6
1.2.2 Accumulated Organic Wastes and Pore Water Exchange.....	6
1.2.3 Environmental Loading .....	7
1.2.4 Stocking Density .....	9
1.3 THE GENUS <i>HALIOTIS</i> .....	10
1.3.1 Abalone Culture.....	12
1.4 FOCUS OF THE CURRENT STUDY.....	14
<b>CHAPTER 2 - Intermittent Exposure To Low Dissolved Oxygen And Elevated Ammonia.....</b>	<b>19</b>
2.1 INTRODUCTION.....	19
2.2 METHODS & MATERIALS .....	21
2.2.1 Experimental System.....	21
2.2.2 Experimental abalone.....	21
2.2.3 Growth trial.....	22
2.2.4 Pulse exposures .....	23
2.2.5 Water quality .....	24
2.2.6 Challenge exposure .....	25
2.2.7 Statistical analysis .....	25
2.3 RESULTS.....	25
2.3.1 Behavioral observations .....	25
2.3.2 Mortality.....	26
2.3.3 Water quality .....	27
2.3.4 Growth data.....	27
2.4 DISCUSSION.....	27
2.5 CONCLUSION .....	32
<b>CHAPTER 3 - Chronic Exposure To Low Dissolved Oxygen Levels Combined With Elevated                     Environmental Ammonia Concentrations .....</b>	<b>38</b>
3.1 INTRODUCTION.....	38
3.2 METHODS & MATERIALS .....	40
3.2.1 Bioassay System.....	40
3.2.2 Experimental Abalone .....	41
3.2.3 Growth Trial.....	42
3.2.4 Water Quality .....	43
3.2.5 Statistical Analysis.....	44
3.3 RESULTS.....	45
3.3.1 Water Quality .....	45
3.3.2 Mortality.....	45
3.3.3 Growth data.....	46
3.4 DISCUSSION.....	47
3.5 CONCLUSIONS .....	53
<b>CHAPTER 4 - Effect Of Chronic And Acute Exposure To Environmental Ammonia On</b>	



<b>Haemolymph Ions .....</b>	<b>59</b>
4.1 INTRODUCTION.....	59
4.2 METHODS & MATERIALS .....	61
4.2.1 <i>Acute Exposure</i> .....	62
4.2.2 <i>Haemolymph Sampling</i> .....	62
4.2.3 <i>Effect of Starvation on Haemolymph Ions</i> .....	63
4.2.4 <i>Statistical Analysis</i> .....	63
4.3 RESULTS.....	64
4.3.1 <i>Effects of Starvation</i> .....	64
4.3.2 <i>Haemolymph pH and Calcium</i> .....	65
4.3.3 <i>Haemolymph Magnesium, Potassium &amp; Sodium</i> .....	65
4.3.4 <i>Haemolymph Copper</i> .....	67
4.3.5 <i>Haemolymph Chloride</i> .....	67
4.4 DISCUSSION.....	68
4.4.1 <i>Haemolymph pH and Calcium</i> .....	70
4.4.2 <i>Haemolymph Magnesium, Potassium &amp; Sodium</i> .....	72
4.4.3 <i>Haemolymph Chloride</i> .....	75
4.4.4 <i>Haemolymph Copper</i> .....	75
4.5 CONCLUSIONS .....	76
<b>CHAPTER 5 - Stocking Density And Refuge Provision In Relation To Growth</b>	
<b>And Water Quality .....</b>	<b>91</b>
5.1 INTRODUCTION.....	91
5.2 METHODS AND MATERIALS.....	93
5.2.1 <i>Experimental system</i> .....	93
5.2.2 <i>Experimental protocol</i> .....	94
5.2.3 <i>Surface Area Calculations</i> .....	94
5.2.4 <i>Experimental animals</i> .....	95
5.2.5 <i>Food Conversion Ratio</i> .....	95
5.2.6 <i>Tank maintenance</i> .....	96
5.2.7 <i>Water quality</i> .....	96
5.2.8 <i>Effect of Current Speed on Diet Composition</i> .....	97
5.2.9 <i>Statistical analysis</i> .....	98
5.3 RESULTS.....	98
5.3.1 <i>Mortality</i> .....	99
5.3.2 <i>Water quality</i> .....	99
5.3.3 <i>Growth Data</i> .....	99
5.3.5 <i>Consumption and Food Conversion Ratio</i> .....	100
5.3.6 <i>Effect of current speed on diet composition</i> .....	100
5.4 DISCUSSION.....	101
5.5 CONCLUSION .....	107
<b>CHAPTER 6 - Stocking Density And Refuge Provision In Relation To Abalone Behaviour.....</b>	<b>120</b>
6.1 INTRODUCTION.....	120
6.2 METHODS & MATERIALS .....	122
6.2.1 <i>Surface Area Calculations</i> .....	122
6.2.2 <i>Behavioural Observations</i> .....	122
6.2.3 <i>Incidence of Shell Damage</i> .....	123
6.2.4 <i>Statistical Analysis</i> .....	123
6.3 RESULTS.....	123
6.3.1 <i>Changes in Submerged Surface Area and Effective Foraging Area</i> .....	123
6.3.2 <i>Distribution</i> .....	123
6.3.3 <i>Behavioral Observations</i> .....	124
6.3.4 <i>Water Currents</i> .....	125
6.3.5 <i>Shell Damage</i> .....	125

6.4 DISCUSSION.....	125
6.5 Conclusions .....	129
<b>CHAPTER 7 - Temporal Changes In Water Quality .....</b>	<b>158</b>
7.1 INTRODUCTION.....	158
7.2 METHODS & MATERIALS .....	160
7.2.1 <i>Experimental diets</i> .....	161
7.2.2 <i>Water quality</i> .....	162
7.2.3 <i>Experimental Protocol</i> .....	162
Bulk water quality (BWQ) under normal flow conditions (T=0).....	163
BWQ after an interruption to water exchange (T=1, whole tank metabolism).....	163
Sediment-enriched bulk water (SEBW) .....	163
7.2.4 <i>Statistical Analysis</i> .....	164
7.3 RESULTS.....	164
7.3.1 <i>Diet Composition</i> .....	164
7.3.2 <i>Mortality Data</i> .....	165
7.3.3 <i>Temperature data</i> .....	165
7.3.4 <i>BWQ during normal water exchange (T=0)</i> .....	165
7.3.5 <i>Effect on BWQ after an interruption in water exchange (T=1, whole tank metabolism)</i> ...	166
7.3.6 <i>SEBW</i> .....	167
7.4 DISCUSSION.....	167
7.4.1 <i>BWQ (T=0 &amp; whole tank metabolism)</i> .....	168
7.4.2 <i>SEBW</i> .....	170
7.4.3 <i>Effect of Diet Composition</i> .....	171
7.5 CONCLUSIONS .....	172
<b>CHAPTER 8 - Nitrogen And Oxygen Budgets For Experimental Abalone Systems.....</b>	<b>184</b>
8.1 INTRODUCTION.....	184
8.2 METHODS .....	186
8.2.1 <i>Nitrogen Budgets</i> .....	186
Trial 1 (September-November 1998) .....	186
Trial 2 (December 1997-May 1998) .....	188
8.2.2 <i>Oxygen Budget</i> .....	189
Trial 1 (Phase 2).....	190
Trial 2.....	190
8.3 RESULTS.....	191
8.3.1 <i>Trial 1</i> .....	191
Composition of diets, abalone tissue and organic wastes.....	191
Nitrogen budget .....	191
Oxygen Budget .....	192
8.3.2 <i>Trial 2</i> .....	192
Organic waste production .....	192
Nitrogen budget .....	192
Oxygen budget.....	192
8.4 DISCUSSION.....	193
8.4.1 <i>Nitrogen Budgets</i> .....	193
8.4.2 <i>Oxygen Budgets</i> .....	196
8.5 CONCLUSIONS .....	198
<b>Chapter 9 - General Discussion.....</b>	<b>209</b>
9.1 IMPACTS OF ELEVATED ENVIRONMENTAL AMMONIA COMBINED WITH LOW DISSOLVED OXYGEN LEVELS .....	210
9.2 STOCKING DENSITY, REFUGE PROVISION & ABALONE BEHAVIOUR .....	213
9.3 OXYGEN BUDGETS & FACTORS AFFECTING WATER QUALITY .....	215
9.4 NITROGEN BUDGETS .....	217
9.5 CONCLUSIONS .....	218
<b>BIBLIOGRAPHY .....</b>	<b>223</b>

# CHAPTER 1

## General Introduction

Throughout history humans have harvested the aquatic environment in order to supplement terrestrial food sources. Simple harvests of aquatic plants and animals are still an important dietary component in rural third world economies, while in more developed areas methods have been developed for increasing the production from aquatic systems. While there is evidence of aquaculture being practiced in Japan and China between 1,000 and 2,000 BC, the first known written records of aquaculture date from China in 460 BC (Stickney 1994). There are also hieroglyphic depictions of what appears to be tilapia culture in the tombs of Egyptian Pharaohs (Stickney 1994). Modern intensive culture methods allow for the production of  $>400 \text{ kg.m}^{-3}$  ( $> 7,500 \text{ kg.ha}^{-1}$ ) compared with  $<1 \text{ kg.m}^{-3}$  ( $<100 \text{ kg.ha}^{-1}$ ) for low input extensive systems common in third world rural communities (Wedemeyer 1996; Stickney 1994). Development of these high yields has been underpinned by advances in production technology and the development of nutritionally complete formulated diets.

Initially, the interest in intensifying the production of natural systems was purely to provide alternative food sources or supplement wild stocks. In modern times, other factors have also led to an increasing interest in the intensive culture of many marine species: over-exploitation of wild populations leading to declining or closed fisheries, modern marketing forces developing markets for premium quality products not available from the wild populations, the increasing popularity of non-traditional and ethnic foods, and the potential to extend product availability past the natural season. In recent decades, advances in disciplines such as biology, endocrinology, nutrition and production technology have allowed for the high density culture of a wide range

of species, including abalone. Each species has its own particular set of conditions in terms of water quality and husbandry which optimize growth and health in commercial systems, and this study was aimed at elucidating some of these parameters for blacklip and greenlip abalone. There are some fundamental concepts which apply to intensive aquaculture in general, which are reviewed below before presenting a more focused discussion on abalone culture.

### 1.1 Modern Intensive Aquaculture, Growth Rates and Water Quality

In order for stock health and optimal growth rates to be maintained under the unnaturally high densities attained by intensive aquaculture systems, the aquaculturist needs to be aware of what factors influence water quality for the species being cultured. While there are a wide range of variables known to affect growth, elevated ammonia and low dissolved oxygen levels are commonly reported as the most likely to affect stock health and production in intensive aquaculture (Colt & Armstrong 1981; Fivelstad *et al.* 1991; Allan *et al.* 1995; Kikuchi 1995; Forsberg 1996).

In solution, ammonia is present as both ionised ( $\text{NH}_4^+$ ) and un-ionised ( $\text{NH}_3$ ) forms, with the balance between the two controlled by temperature, ionic strength and pH. Historically, the un-ionised fraction has been associated with toxic effects due to the ease of transfer of small neutral molecules such as  $\text{NH}_3$  across lipid-based branchial membranes, and the fact that it is the dominant form present in most aquatic environments (Bower & Bidwell 1978; Thurston *et al.* 1981a; Wilkie 1997). However, ionised ammonia may have more impact on marine species as their branchial membranes are more permeable to cations than those of freshwater species, due to leaky paracellular junctions (Evans & Cameron 1989; Wilson & Taylor 1992; Wilkie 1997).

Nitrogenous excretions resulting from catabolism of ingested protein is the main source of ammonia in most culture systems, with 50-90% of the total nitrogen ingested excreted as ammonia in salmonids (Fivelstad *et al.* 1990; Jayaram & Beamish 1992; Mommsen & Walsh 1992; Forsberg 1997), crustaceans (Wajsbrodt *et al.* 1989; Chen & Cheng 1993) and prosobranch gastropods such as abalone (Bishop *et al.* 1983; Peck *et*

*al.* 1987). Urea forms the bulk of the remaining nitrogen excreted, and is generally excreted at a constant rate, although some authors have shown that some fish species alter urea excretion in response to diet composition and starvation (Jayaram & Beamish 1994; Kikuchi 1995; Dosdat *et al.* 1996). Ammonia excretion in aquatic animals is influenced by a range of factors, including reproductive maturation (Bishop *et al.* 1983; Navarro & Torrijos 1994), smoltification (Bergheim *et al.* 1991) seasonal factors and body size (Gabbot 1983; Wajsbrodt *et al.* 1989). Feeding has a well documented influence on ammonia excretion in fish (Fivelstad *et al.* 1990; Jayaram & Beamish 1992; Kikuchi 1995; Forsberg 1997), crustaceans (Wajsbrodt *et al.* 1989; Taboada *et al.* 1998) and molluscs (Bishop *et al.* 1983), as protein from ingested ration is catabolized and leads to transient increases in plasma ammonia levels (Dosdat *et al.* 1996). The level and quality of protein in the diet will affect ammonia excretion (Fleming *et al.* 1996; Schmitt & Santos 1998; Taboada *et al.* 1998), as will the use of protein for energy if the protein/energy ratio is not suitably balanced (Degani & Levanon 1988; De Silva *et al.* 1991; Jayaram & Beamish 1992). The use of dietary lipid to spare protein has been demonstrated in a range of species including tilapia (De Silva *et al.* 1991), salmonids (Kaushik & Teles 1985), penaeids (Koshio *et al.* 1993) and abalone (Britz & Hecht 1997). Optimising the protein component of formulated diets in this manner will reduce ammonia excretion and the nitrogen load on the environment.

A wide range of physical and physiological effects, extensively reviewed in several papers (eg. Colt & Armstrong 1981; Meade 1985; Russo 1985; Russo & Thurston 1991; Mommsen & Walsh 1992), have been attributed to elevated environmental ammonia levels. Documented toxic effects of ammonia exposure on a range of aquatic species include reduced food consumption (Rasmussen & Korsgaard 1996; Person-LeRuyet *et al.* 1997; Harris *et al.* 1998a), behavioural changes (Arillo *et al.* 1981; Rasmussen & Korsgaard 1996), haematological disturbances (Dabrowska & Wlasow 1986) and disruption of metabolic and enzymatic pathways (Arillo *et al.* 1981; Jeney *et al.* 1992). Growth rate depression arising from a combination of a reduction in food consumption combined with a decrease in assimilation efficiency

has been documented for turbot (*Scophthalmus maximus*) exposed to environmental ammonia (Rasmussen & Korsgaard 1996). There is also clear evidence that ammonia can alter the conformation of enzymes such as phosphofructokinase and stimulate certain glycolytic pathways in salmonids (Sousa & Meade 1977), tilapia (Begum 1987) and molluscs (Chetty & Indira 1995).

Ammonia exposure has also been linked to increases in serum pH, both from metabolic and respiratory origins (Sousa & Meade 1977; Wilson & Taylor 1992; El-Shafey 1994; Schmit & Santos 1999). As the loading and unloading of haemoglobin is very sensitive to pH, described by the Bohr and Root effects, this will affect normal respiratory function and in teleosts leads to a reduction in the capacity of haemoglobin to load oxygen at the gills and unload it at the tissues.

Sublethal exposure to low dissolved oxygen levels is not directly toxic, but affects growth by limiting the capacity for aerobic metabolism (Brett 1979; Seidman *et al.* 1985; Sprague 1985). When alternative catabolic pathways are used with electron donors other than oxygen less energy is available for somatic growth, which has biological consequences for the organism and economic consequences for the aquaculturist (Seidman *et al.* 1985; Rosas *et al.* 1999). Oxygen consumption and oxygen budgets are of interest to the aquaculturist because of the sensitivity of many cultured species to low dissolved oxygen levels (reviewed in Harris *et al.* 1999a). Increases in oxygen consumption as a result of feeding (specific dynamic action, SDA) are well documented for fish (Forsberg 1997) and gastropods (Carefoot 1987; Low 1995). Active species such as salmonids tend to consume oxygen at a relatively constant rate throughout a given 24 hour period (Fivelstad *et al.* 1990). Foraging activity in nocturnally active gastropods such as abalone (*Haliotis* spp.) and loco (*Concholepas concholepas*) results in diurnal patterns of oxygen consumption (Peck *et al.* 1987; Jan *et al.* 1981; Navarro & Torrijos 1994; Donovan & Carefoot 1998; McBride *et al.* 2001).

While the effect of ammonia and low dissolved oxygen are commonly investigated as individual toxicants, it is unlikely that they will be present alone in an intensive culture system (Millamena 1990; Allan *et al.* 1995). In pond systems, there

are substantial changes in pH, DO and ammonia both diurnally, as the level of photosynthetic activity changes with light intensity, and over longer periods as algal blooms develop and crash (Krom *et al.* 1985a). However, the effects of elevated environmental ammonia in conjunction with low dissolved oxygen has received scant attention in the literature. The studies which are available indicate that the toxic effects of environmental ammonia are increased in the presence of low oxygen tensions in fish (Downing & Merkens 1955; Merkens & Downing 1957; Lloyd 1961; Thurston *et al.* 1981c; Alabaster *et al.* 1983; Wajsbrot *et al.* 1991; Tudor *et al.* 1994) and crustaceans (Allan & Maguire 1990; Wajsbrot *et al.* 1990; Chen & Nan 1992a).

Water quality in intensive culture systems is also unlikely to be static, with ammonia and dissolved oxygen changing diurnally in response to feeding activity and post-prandial metabolism. The effects of such changes, and at what point they begin to have an impact on growth or stock health, have not been widely studied. Poxton & Allouse (1987) showed that ammonia levels in a recirculating system holding turbot change over both short term (24 hour) and longer term (5-7 days) cycles, related to feeding activity and feeding rate. Although the authors concluded that the magnitude of the changes was unlikely to cause growth reductions or affect health, there are no data available on the long term effects of sub-lethal fluctuations in ammonia or DO. Soderberg (1985) reported that rainbow trout exposed to episodes of elevated ammonia concentrations developed gill lesions unlikely to have been caused by the baseline conditions.

Invertebrate species which are naturally exposed to periods of low DO are biochemically and physiologically equipped to use anaerobic metabolism during hypoxic or anoxic episodes (Brix *et al.* 1979; Taylor & Spicer 1987; Johnson & Uglow 1987; McMahon 1988; Storey & Storey 1990; Carroll & Wells 1995; Wells *et al.* 1998a,b). These episodes are likely to occur in the inter-tidal/sub-littoral zone (Taylor & Spicer 1987; Johnson & Uglow 1987; McMahon 1988), which is inhabited by some commercially important species of abalone. Many invertebrate species including gastropods are also well adapted to the functional hypoxia which develops from short burst of intense activity (such as escaping from a predator) or sustained

exercise such as locomotion in gastropods (Gäde 1988; Gäde & Grieshaber 1986; Carroll & Wells 1995; Wells *et al.* 1998a,b).

## **1.2 Biotic and Abiotic Factor influencing water quality**

### ***1.2.1 Influence of Bulk Water Exchange***

Water exchange has both biological and economic ramifications for intensive aquaculture (Fivelstad 1988; Wajsbrodt *et al.* 1989; Fivelstad *et al.* 1991; Allan & Maguire 1993). Influent water dilutes toxic metabolites such as ammonia, and is the primary source of oxygen (Handy & Poxton 1993; Kochba *et al.* 1994). Water exchange rate has been shown to have significant effects on water quality in prawn ponds (Hopkins *et al.* 1993). However, the cost of pumping and maintaining a water supply can be a substantial part of the production costs. Biomass production and economic return are thus linked to water exchange, within the biological limits of the species.

### ***1.2.2 Accumulated Organic Wastes and Pore Water Exchange***

Organic wastes, consisting primarily of uneaten food and faecal material, are an unavoidable component of intensive aquaculture, as a result of high stocking densities and the use of formulated diets (Gowen & Bradbury 1987; Poxton 1991; Roden 1997). In pond systems, there is also a contribution from bottom soil and phytoplankton (Smith 1996). These wastes are important in terms of the net environmental load from the facility, and also because of potential impacts on bulk-water quality as microbial activity degrades the organic material (Santschi *et al.* 1990; Colman & Jacobson 1991; Holmer 1991; Masuda & Boyd 1994). In pond systems, these wastes can leach nutrients back into the water column and support phytoplankton blooms (Krom *et al.* 1985a,b; Schroeder 1987). In some cases this is managed as a supplementary food source either directly, or after microbial degradation of the organic wastes (Schroeder 1987).

Once microbial degradation proceeds to the point where the diffusion of oxygen into the wastes is inadequate to meet the oxygen demand of the sediment, the oxidative processes continue using other electron donors such as iron, sulphur and



manganese (Val Klump & Martens 1981; Colman & Jacobson 1991; Holmer 1991; Masuda & Boyd 1994). Similar processes have been described in organic-rich oceanic sediments (McCaffrey *et al.* 1980; Val Klump & Martens 1981; Santschi *et al.* 1990). In marine sediments, the reduction of sulphate to sulphide is the primary sink for electrons for the degradation of organic matter in the absence of oxygen (Val Klump & Martens 1981; Raa & Liltved 1991; Suplee & Cotner 1996). Sediment oxygen demand (SOD) can be 50-70% of the oxygen demand of pond systems (Colman & Jacobson 1991; Suplee & Cotner 1996), of which a substantial component is re-oxidation of sulphide (Suplee & Cotner 1996). Growth reductions have been reported in fish and prawn ponds as a result of anaerobic sediments developing, although the exact mechanism remains to be clarified (Avinmelech & Zohar 1986; Allan *et al.* 1995).

Water quality within the interstitial matrix of the organic wastes (pore water) is influenced by the nature of the organic material accumulating and by the rate of accumulation, but is generally acidic, chemically reduced, oxygen depleted and loaded with other products of microbial decomposition (Masuda & Boyd 1994; Roden 1997).

Transfer in either direction across the sediment:water interface (i.e. between bulk water and pore water) is controlled primarily by diffusion through the diffusive boundary layer (DBL) and bioturbation by benthic fauna (Schroeder 1987; Santschi *et al.* 1990; Colman & Jacobson 1991). There is an inverse relationship between the rate of diffusion and thickness of the diffusive boundary layer, which in turn is controlled by current flow in the water column and surface roughness of the sediment layer. Water quality adjacent to the organic sediments is therefore likely to differ from the bulk water (Schroeder 1987; Roden 1997).

### ***1.2.3 Environmental Loading***

The potential for modern intensive aquaculture to have an impact on the wider environment has become apparent through the experience of European and Scandinavian countries (Gowen & Bradbury 1987; Beveridge *et al.* 1991; Woodward *et al.* 1992). In conjunction with an increasing environmental awareness, the

environmental impact of intensive aquaculture has developed into a contentious issue in recent years.

Production of the biomass generated by intensive aquaculture requires substantial organic inputs in the form of formulated diets, which can potentially alter the environment both within the facility and in the receiving waters (Gowen & Bradbury 1987). The potential for water quality to become degraded to the point of affecting production or stock health concerns the aquaculturist, while the environmentalist is more interested in changes involving the local ecosystem on a larger scale (Poxton 1991; Raa & Liltved 1991). These positions are not necessarily mutually exclusive.

Of the total amount of formulated diet supplied in most aquaculture operations, 10-20% is not consumed, and 20-30% of ingested dry matter is passed as faeces, which leaves 50-70% available for growth and production of the harvested product (Gowen & Bradbury 1987; Beveridge *et al.* 1991). Similar values apply to nitrogen, with even less of the ingested total assimilated due to excretion of up to 50% of the ingested nitrogen as ammonia (Handy & Poxton 1993). The percentage of nitrogen assimilated into tissue is around 20-30% for most species examined (18-27% for penaeid prawns (Briggs & Funge-Smith 1994; Funge-Smith & Briggs 1998); 36% for *Sparus aurata* (Krom *et al.* 1985b); 22.3% for striped bass (*Morone saxatilis*, Daniels & Boyd 1989); 27-28% for salmonids (Hall *et al.* 1992)).

Mass balances for nutrients such as nitrogen and phosphorus have been constructed for a range of systems and species, to identify where the various nutrients added to the system are distributed (Gowen & Bradbury 1987; Beveridge *et al.* 1991; Bergheim & Åsgård 1996; Cho & Bureau 1997). Nitrogen budgets for salmonids show that a substantial part of the nitrogen loading in the effluent is soluble, and thus difficult to control by directly treating the effluent (Bergheim & Åsgård 1996). Since many marine environments are low in nitrogen, such nitrogen-enriched effluents may have substantial impacts in marine systems (Hall *et al.* 1992; Cripps & Kelly 1996). Nitrogen budgets allow both regulators and aquaculturists to identify the net load on the environment, and which components can be controlled or reduced (Briggs &

Funge-Smith 1994). Retaining particulate waste can reduce the net environmental load of particulate matter and nitrogen, provided the collected material is removed from the site (Bergheim & Åsgård 1996; Cripps & Kelly 1996). Diet formulation is a central component in minimizing aquaculture waste, with use of more digestible ingredients and optimization of the protein/energy ratios reducing pollution from salmonid farms in Scandinavia and Europe (Cho *et al.* 1991; Bergheim & Åsgård 1996; Cho & Bureau 1997).

#### ***1.2.4 Stocking Density***

As one of the prime determinants of commercial viability in intensive aquaculture systems, the relationship between stocking density, growth and biomass production for a wide range of both vertebrate and invertebrate species has received considerable attention in the literature (Kushnirov & Degani 1991; Allan & Maguire, 1992; Chaitanawisuti & Kritsanapuntu 1997; Wagner *et al.* 1997; Hossain *et al.* 1998; Verhoef & Austin 1999). Each species has an optimum stocking density, which is dependent on the ecology and behaviour of that species. For example, optimum growth rates in pelagic species such as European sea bass (*Dicentrarchus labrax*) require relatively high stocking densities in order to allow natural schooling behaviour (Papoutsoglou *et al.* 1998). Stocking density in benthic species such as African catfish (*Clarias gariepinus*) is limited by aggressive behaviour including cannibalism (Hossain *et al.* 1998).

Traditional units for stocking density ( $\text{kg.m}^{-3}$ ) are not necessarily applicable to benthic species such as abalone and prawns where volume is not as important as surface area. Prawn densities have been quoted as either prawns or  $\text{biomass.m}^{-2}$  (Allan & Maguire 1992), as have abalone densities (MacShane & Naylor 1995; Marsden & Williams 1996; Capinpin *et al.* 1999). Describing the carrying capacity for abalone is also complicated by the use of refuges, and a more precise expression would be biomass per unit submerged surface area (Mgaya & Mercer 1995), which is the total submerged surfaced area available to the abalone. The optimum stocking

density for commercial production may not be the same density that promotes the fastest growth rates. Biomass gain for the system increases with density, and thus the cost per kg of biomass will decline (Allan & Maguire 1992; Mgaya & Mercer 1995).

### 1.3 The Genus *Haliotis*

Abalone (genus *Haliotidae*) are benthic gastropods found in most coastal waters around the world (Hahn 1989b; Voltzow 1994). They are considered a relatively primitive genus compared to other gastropod classes (Russell-Hunter 1983; Voltzow 1994), being placed in the order Archeogastropoda within the sub-class Prosobranchia of the class Gastropoda (Trueman & Clarke 1985; Voltzow 1994).

The eggs are externally fertilized and hatch within 24 hours. The pelagic larvae (veligers) do not feed directly, although there is evidence that they may absorb organic molecules from the water column (Wells 2003). After a relatively short larval cycle, the larvae settle and become post-larva, developing the adult form and starting to produce a shell. The first respiratory pore is formed approximately 1 month after settlement, at which point they are generally called juveniles (Hahn 1989c). The larvae settle on diatoms or coralline algae, with different substrates being preferred by different species. As the juveniles grow, they graze on diatoms and macro-algae. The gills and excretory organs are located in a cavity formed by the mantle (pallial cavity), which lies along one side of the pedal muscle, underneath a series of holes in the shell (respiratory pores). Various researchers have shown that the shape, position and size of the holes induce water movement through the pallial cavity at very low external current speeds both in abalone (Voltzow 1983; Tissot 1992) and other benthic gastropods (Murdock & Vogel 1978).

Haliotids are predominantly active at night and quiescent during the day. The preferred habitat for the day time inactive period ranges from crevices or under boulders for juveniles and cryptic species to more exposed areas for adults in some species (Poore 1972; Shepherd 1973; Hahn 1989). Most species are found in aggregations with the density dependent on species (Poore 1972; Shepherd 1973; Hahn 1989b).

The bulk of abalone soft tissue is the pedal muscle, which has several functions. As well as elevating the shell during foraging activity, and clamping it tightly to the substrate during the quiescent period, it is also involved in locomotion and righting the abalone should it be turned over on the shell (Gäde 1988; Wells *et al.* 1998a). While most authors describe this as one muscle, Gäde (1988) divides it into the adductor muscle (holding the shell down, righting movements) and foot muscle (locomotion). This muscle may form more than 60% of the live weight of the animal (Jorgensen *et al.* 1984) and requires substantial amounts of oxygen for aerobic function, especially when it is considered that abalone are among the fastest locomoting gastropods (Wells *et al.* 1998a).

While haemocyanin is present in abalone serum, and may bind up to 80% of the available oxygen (Ainslie 1980), it is likely that this would be unable to maintain an adequate supply of oxygen to the pedal muscle for extended periods of foraging activity, or even short term bursts of intense activity (Ellington 1983; Gäde 1983; Wells *et al.* 1998a), resulting in the development of functional hypoxia. The pedal muscle in abalone receives a much lower proportion of the blood flow than other more important tissues such as heart and digestive gland (Jorgensen *et al.* 1984) and abalone are also known to restrict blood flow to the pedal muscle during periods of exercise (Russell & Evans 1989). In conjunction with the large sinuses which are found in the abalone circulatory system and a relatively large blood volume (Jorgensen *et al.* 1984; Russell & Evans 1989), this will maintain a reserve of oxygen for the sensitive tissues, such as kidney and heart, during hypoxia and delay the development of hypoxic conditions in these tissues (Wells *et al.* 1998a). This supply is further extended during hypoxia by a reversal of the Bohr and Root effects found in vertebrate haemoglobin. This reverse effect has been demonstrated in haemocyanin from a prosobranch gastropod (*Buccinum undatum*, Brix *et al.* 1979) and abalone (Ainslie 1980; Wells *et al.* 1998a), and means that the haemocyanin molecule will bind oxygen more strongly as pH declines and carbon dioxide levels increase during hypoxia (Wells *et al.* 1998a).

It is likely that hydrolysis of arginine phosphate would supply ATP in the early stages of limited oxygen availability in molluscan foot muscle (Gäde 1983; Gäde &

Ellington 1983; Hochachka *et al.* 1983; Wells *et al.* 1998a). Reserves of this substrate would rapidly become depleted, at which point glycogen and aspartate become the fuel for anaerobic glycolysis to maintain ATP production for fueling metabolic processes (Gäde 1983; Gäde & Ellington 1983; Gäde 1988). Under aerobic conditions, the Krebs cycle (also known as the TCA cycle) would regenerate  $\text{NAD}^+$  from NADH for glycolysis (McGilvery & Goldstein 1983). Once oxygen is either limited or not available, the regeneration of NADH is maintained by the reductive condensation of pyruvate with an amino acid such as alanine, arginine or glycine (Fields 1983; Gäde 1988; Carroll & Wells 1995), resulting in the formation of opines. In abalone, taurine is used to produce tauropine (Gäde 1988; Ryder *et al.* 1994; Wells & Baldwin 1995; Baldwin *et al.* 1992). D-lactate is also produced (Ryder *et al.* 1994), mainly in the foot muscle rather than the adductor muscle (Gäde 1988; Wells & Baldwin 1995). The production of tauropine can be shown thermodynamically to maintain a lower  $\text{NADH}/\text{NAD}^+$  ratio than formation of lactate as in vertebrate systems, which will optimize the yield of ATP from anaerobic glycolysis (Fields 1983; Gäde 1988). These end products will also have less impact on osmotic pressure than the conversion of glycogen to lactate (Fields 1983).

It is generally considered that tauropine formation predominates during functional hypoxia, and lactate is the result of environmental hypoxia (Gäde 1983, 1988; Carroll & Wells 1995; Wells & Baldwin 1995; Wells *et al.* 1998a,b).

### ***1.3.1 Abalone Culture***

Having a relatively large and edible foot muscle, which may be more than 60% of the live weight (Jorgensen *et al.* 1984), and being abundant in shallow water (commonly the sub- and inter-tidal zones) abalone have been harvested for thousands of years on several continents (Shepherd 1973; Shepherd *et al.* 1992; Voltzow 1994). Asian consumers, traditionally substantial consumers of aquatic products, have fished abalone from wild stocks for centuries (Shepherd *et al.* 1992), and are the main market for both wild and cultured abalone (Oakes & Ponte 1996; Hone & Maguire 1996; Gordon & Cook 2001).

The increasing demand for this commodity with its unique organo-leptic qualities, particularly in Asian countries, has led to unsustainable pressure on wild stocks from over-exploitation, fishery mismanagement and poaching. Very few commercially fished stocks around the world have not declined or collapsed (for reviews, see Shepherd *et al.* 1992; Cook 2001). As with many other species, this has led to the development of intensive culture techniques in order to ease the pressure on natural populations, to provide consistent supplies of premium quality products and to develop products not available from the wild fishery (Fleming & Hone 1996; Oakes & Ponte 1996; Jarayabhand & Paphavasit 1996; Gordon & Cook 2001). This was the impetus behind the beginnings of research into propagation of abalone in Japan, China and Taiwan in the 1950's, with the result that abalone culture is now well established in several countries such as China, Japan Australia, South Africa and North America (Shepherd *et al.* 1992; Fleming & Hone 1996; Fleming *et al.* 1996; Cook 2001). Many other countries such as Chile, Iceland, Ireland, Israel, Thailand and at least one Arab state are trialing abalone culture (S.A. Shepherd 2001, pers. comm.). A wide range of nutritional, husbandry and environmental issues have been investigated and reviewed by a number of researchers (Uki & Watanabe 1992; Fleming & Hone 1996; Fleming *et al.* 1996; Harris 1999; Harris *et al.* 1998a, 1999a,b).

Abalone are cultured in a range of systems including land-based tanks or race-ways and mesh containers on submerged longlines (reviewed in Hahn 1989a; Aviles & Shepherd 1996; Hindrum *et al.* 1995, 1996; Loipersberger 1997; Grove-Jones 1995). Some tanks are designed to be self cleaning, by preventing the accumulation of organic waste (Grove-Jones 1995; Loipersberger 1997). Systems in which the organic wastes accumulate have some similarities to pond systems used in fish or prawn culture, although regular removal of accumulated wastes prevents the build up of nutrients and trophic levels characteristic of these ponds (Kochba *et al.* 1994; Funge-Smith & Briggs 1998).

Chronic bioassays have shown that greenlip abalone are sensitive to low pH, low dissolved oxygen and elevated ammonia levels (see Table 1.1). However, given that in the wild abalone can be found in dense aggregations and in crevices, it would be

expected that they would be periodically exposed to low dissolved oxygen and elevated ammonia. As discussed gastropods including abalone are also known to be biochemically suited to cope with functional hypoxia as well, which would also provide a degree of protection against environmental hypoxia.

The relationship between stocking density and growth for abalone has been studied both in the wild (MacShane & Naylor 1995) and in a range of experimental-scale culture systems (Mgaya & Mercer 1995; Moore & Hone 1995; Marsden & Williams 1996; Capinpin *et al.* 1999). In abalone, growth rate generally declines as density increases, which has also been reported for another benthic gastropod, the spotted Babylon (*Babylonia areolata*, Chaitanawisuti & Kritsanapuntu 1997). A significant component of this reduction is likely to be increased competition for food or primary attachment space (Dourois 1987; Mgaya & Mercer 1995). The potential for refuges to improve growth by increasing the primary attachment area has received much less attention. Refuges are used in culturing a number of aquaculture species, primarily for shelter and to control aggressive or hierarchical behaviour (Hossain *et al.* 1998; Kushnirov & Degani 1991). Abalone are different in that while provision of refuges does provide shelter, their benthic, cryptic nature means that provision of refuges may also lead to improvements in growth through increasing the submerged surface area of the tank (SSA, the substrate area available for the abalone during the inactive daylight period). Greenlip abalone, the main cultured species in Australia, have been observed in the wild actively seeking refuge in the form of rock crevices (Shepherd 1986a; Shepherd & Partington 1995).

The requirement for a solid substrate means that organic wastes will also accumulate in abalone culture systems, and must be removed by regular cleaning. While these wastes have the potential to degrade water quality, both inside the facility and on a wider scale, this area of investigation has received very little attention.

#### **1.4 Focus of the Current Study**

Over several years, the University of Tasmania has been extensively involved with research on abalone culture as part of a national research program, with a range



of husbandry and management issues having been investigated. This study was aimed at extending some of this earlier research, and also investigating some factors which had not previously been covered.

Figure 1.1 summarises the broad scope of this study. A central focus was on the potential impact of combined exposure to low dissolved oxygen levels in conjunction with elevated ammonia concentrations on growth and survival of blacklip and greenlip abalone, and conditions influencing the development of such combined exposures in commercial grow-out systems.

Chronic bioassays have previously been conducted for a range of water quality variables as individual exposures (Table 1.1), and these showed that abalone are sensitive to low dissolved oxygen and elevated environmental ammonia. However, these investigations did not cover the effects of low dissolved oxygen combined with elevated ammonia, and there are no reports in the literature of such exposure for abalone, or indeed any gastropod mollusc. In this study, the effect on growth of such combined exposures was investigated as an intermittent exposure at different intervals (Chapter 2), and a chronic exposure (Chapter 3). Serum ions were also analysed to study some fundamental physiological effects of chronic exposure to environmental ammonia (Chapter 4).

The requirements for shade and shelter have been addressed in previous research, demonstrating growth improvements if either refuges or shade were provided. This study examined the issue of growth in relation to stocking density and level of refuge provision, (Chapter 5) along with water quality issues. Behavioural aspects in relation to stocking density and refuge provision are developed in Chapter 6.

While it is important to know what factors will influence growth and health, it is just as important to know what factors govern water quality in commercial systems. Some of these factors, namely accumulation of organic wastes, diurnal activity and an interruption to water exchange, are covered in Chapter 7. Chapter 8 uses data generated from Chapters 5 and 7 to develop oxygen and nitrogen budgets for abalone systems.

The investigations presented in this study cover a wide range of husbandry and management issues which will impact on stock health, growth and water quality in abalone grow-out systems. In Chapter 9, the links between the different chapters are discussed as integral components of an overall system which constitute a commercial abalone growout system.

Variable	Blacklip Abalone		Greenlip Abalone	
	Range		Range	
pH	7.93 to 8.46 ≤ 7.46 and ≥ 9.01	Outside this range growth is reduced by ≥ 5%. Mortalities ≥ 45% at these values.	7.78 to 8.77 ≤ 7.16 and ≥ 9.01	Outside this range growth is reduced by ≥ 5%. Mortalities ≥ 18%
Unionised Ammonia			0.041 mg UIA.L <sup>-1</sup>	5% reduction in growth. Above 0.11 mg UIA.L <sup>-1</sup> increased mortality. (Nitrite ≤ 0.13 mg L <sup>-1</sup> )
Dissolved oxygen			≤ 9.1 mg. L <sup>-1</sup> (123%sat) 7.36 mg. L <sup>-1</sup> (96% sat) ≤ 6.2 mg. L <sup>-1</sup> (81% sat)	Growth and mortality the same as at 100% sat. Below this, growth is reduced by ≥ 5%. Mortality > 10%

Table 1.1 Effect on growth of greenlip abalone from alterations in water quality, as indicated in the table. Unless specified, temperature ≈17°C, salinity = 35 ‰. (Harris *et al.* 1997, 1998a, 1999a).

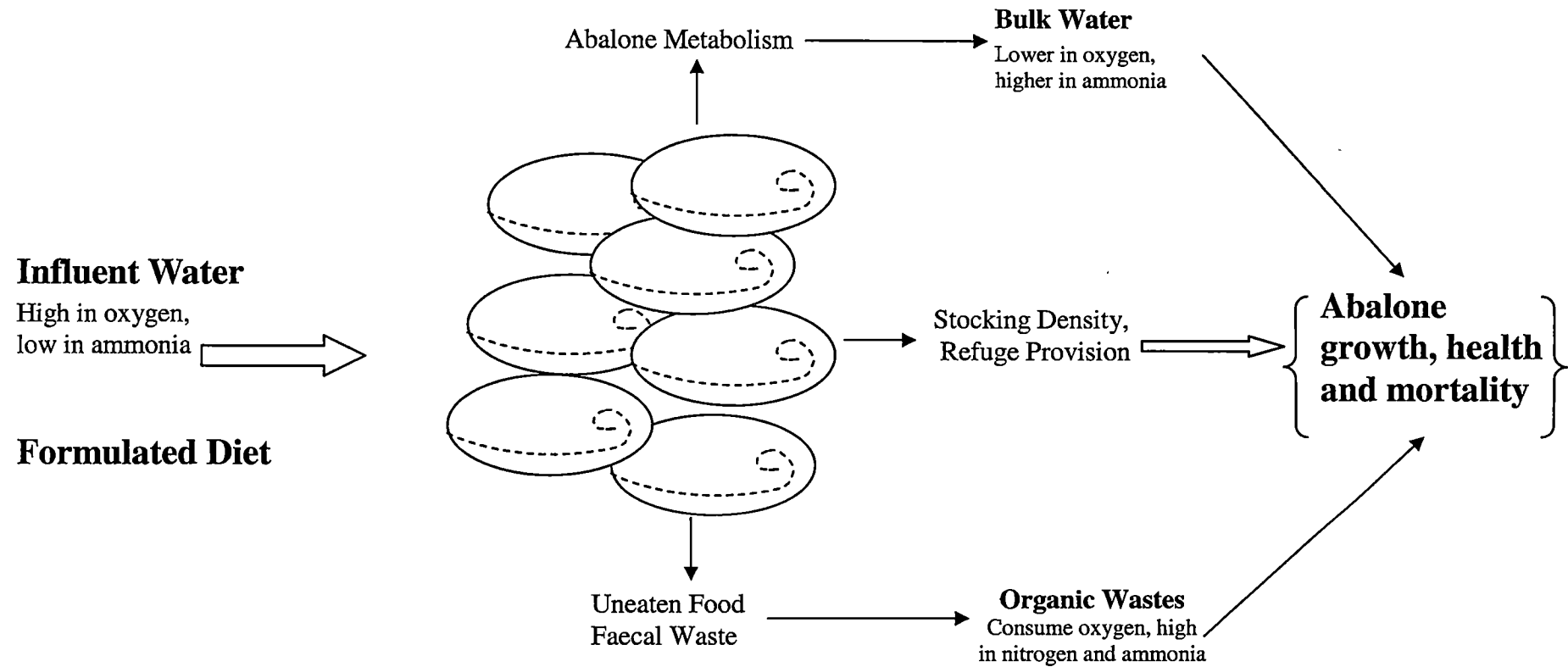


Figure 1.1 Overview of the components in an abalone culture system, and the factors relevant to the present study

## CHAPTER 2

### Intermittent Exposure To Low Dissolved Oxygen And Elevated Ammonia

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Hindrum S.M., Burke C.M., Edwards S.J. & Johns D.R. (2001) Effects of combined exposure to elevated ammonia and low dissolved oxygen levels in greenlip (*Haliotis laevis* Donovan) and blacklip (*H. rubra* Leach) abalone. 1. Growth and mortality data from simulated systems failure. *Journal of Shellfish Research* 20(1): 679-684.

#### 2.1 Introduction

Due to the concomitant demands of aerobic metabolism and production of ammonia as the primary nitrogenous waste product, elevated ammonia concentrations are likely to be found in conjunction with dissolved oxygen levels below saturation in intensive aquaculture (Allan *et al.* 1990; Russo & Thurston 1991; Handy & Poxton 1993; Person-Le Ruyet *et al.* 1997). These factors will become problematic either acutely from an interruption in influent water from some form of systems failure, or chronically from management factors such as overstocking or inadequate removal of organic wastes. As outlined in Chapter 1, the physical and physiological effects of ammonia on a range of fish and invertebrate species are well documented, as are the effects of hypoxia. Invertebrate species which are naturally exposed to periods of environmental hypoxia are biochemically and physiologically adapted to providing energy via anaerobic respiration during such episodes (Hagerman *et al.* 1990; Spaargaren 1990; Storey & Storey 1990). A functional hypoxia is also likely to develop in the pedal muscle of abalone during

exercise, as a result of its large size relative to the capacity of the circulatory system to maintain an adequate supply of oxygenated haemoglobin (Gäde 1988; Wells & Baldwin 1995; Wells *et al.* 1998a,b).

While it is the desire of any aquaculturist to maintain a constant supply of air and water for the species being cultured, forces outside human control will inevitably interrupt or reduce the supply of water during some part of the production cycle. Once the regular supply of air and water has been interrupted or reduced, a rapid decline in water quality can be expected as oxygen consumption continues and metabolites accumulate. In marine aquatic systems, this rapidly becomes problematic because potentially toxic ammonia is the primary nitrogenous metabolite excreted by vertebrate and invertebrate marine species (Colt & Armstrong 1981; Russo & Thurston 1991). In aqueous solution, ammonia exists as a combination of ionized ( $\text{NH}_4^+$ ) and un-ionized forms ( $\text{NH}_3$ ), with the equilibrium between the two being governed by pH, temperature and the ionic strength of the medium (Colt & Armstrong 1981; Meade 1985; Russo & Thurston 1991). Although un-ionized ammonia (UIA) has traditionally been accepted as the predominantly toxic form, there is increasing evidence that the ionized form has a more important role in ammonia toxicity than previously considered (Thurston *et al.* 1981a; Meade 1985; Russo & Thurston 1991). This is particularly evident in marine species, where the osmotic environment means that these organisms have different ion permeabilities than in fresh water (Evans & Cameron 1989; Wilson & Taylor 1992).

Gastropod molluscs such as abalone are biochemically suited to surviving periods of hypoxia or even anoxia, using alternative pathways for the generation of ATP which are not dependent on oxygen (Brix *et al.* 1979; Gäde & Ellington 1983; Storey & Storey 1990), presumably an adaptation to episodes of environmental and functional hypoxia. It is not clear whether molluscs are similarly suited to episodes of elevated ammonia, or low oxygen tensions in combination with elevated ammonia levels. Results from individual bioassays show that abalone are sensitive to chronic exposure to elevated ammonia and low dissolved oxygen levels, with 5% and 50% reductions in growth rates reported at 41 and 158  $\mu\text{g.L}^{-1}$  UIA and 7.36 and 5.91 mg.

$L^{-1}$  DO (Harris *et al.* 1998a, 1999a), which is reported in these studies as more sensitive than a range of aquatic species.

It is important that aquaculturists have an understanding of the sensitivity of the cultured species to changes in water quality that are likely to occur when influent water supply is interrupted for an extended period. Although the body of literature on abalone is increasing, there is a paucity of information on acute or chronic exposure to combinations of altered water quality variables. As well as the possibility of direct mortality, potential stress related complications include secondary infections and growth depression. Repeated episodes may augment such effects or enable acclimation such that the abalone are better able to deal with more severe subsequent exposures.

The aim of this trial was to simulate an extended systems failure by manipulating oxygen and ammonia levels at intervals ranging from once in 42 days to every 7 days. The effect of exposure history on the response to a more severe challenge was also assessed.

## **2.2 Methods & Materials**

### ***2.2.1 Experimental System***

The experimental tanks were circular fibreglass units (diameter = 70 cm., volume = 55 L). The 30 tanks used in the trial were enclosed within an area covered with shade cloth to prevent the development of algal biofilms. Each tank was individually supplied on a flow-through basis with sand-filtered oceanic seawater, drawn from sub-surface intakes on an exposed coastline. The salinity was 34-35 g.L<sup>-1</sup> throughout the trial, and not affected at any stage by freshwater run-off. Aeration was supplied through two 50 mm airstones per tank.

### ***2.2.2 Experimental abalone***

The blacklip abalone were obtained from commercial stock at Swansea, Tasmania, Australia, and were 18-24 months old at the start of the trial. For blacklips, mean length = 42.9±3.6 mm, mean weight = 13.00±4.45 g (± SD, n=600). The greenlip abalone were obtained from commercial stock at Bicheno, Tasmania,

Australia (41°5'S, 148°18'E) and were 24-30 months old at the start of the trial (mean length = 45.8±3.9 mm, mean live weight = 12.21±3.01 g (± SD, n=600)). Both species had been held in 270 L holding tanks for 3-8 weeks before being randomly allocated to individual tanks after being weighed (to 0.01 g) and measured for length (to 0.1 mm, using Vernier calipers). Polyethylene tags (Hallprint, Adelaide, Australia) had previously been applied, with any missing tags replaced using a cyanoacrylate adhesive gel. Abalone were stocked at 40 animals per tank with triplicate tanks of each species per treatment. The treatments were stocked at intervals of 6-7 days, with the abalone being carefully removed from the holding tanks without anaesthesia, using a spatula.

The diet used prior to and during the trial was based on a proprietary commercial formulation (ABCHOW), with the addition of 10% algal meal. The tanks were routinely cleaned every 4-5 days by siphoning the bulk of the organic wastes, spinning the water gently, and then siphoning off the remaining wastes which collected in the center of the tank. The abalone were not exposed to air during cleaning. Fresh food was supplied every 2 days, with the weighed ration for each tank adjusted on an ad libitum basis.

### 2.2.3 Growth trial

The growth trial ran for 47 days, with ambient water quality shown in Table 2.2. At the intervals indicated in Table 2.1, the tanks were nominally exposed to 60% of the ambient dissolved oxygen concentration and 150 µg.L<sup>-1</sup> UIA. The actual conditions during these exposures are given in Table 2.2. The first exposure for Treatment 5 was 4 h; all subsequent exposures were 8 h (starting between 8.30 and 11 am).

Based on lengths and weights for individual animals averaged for each tank, the growth indices given in Figure 2.1 were calculated as follows :

$$\begin{aligned} \text{Specific Growth Rate for Length (SGR-L, \% change.day}^{-1}\text{)} \\ = \{[\ln(L_2) - \ln(L_1)] * 100\} / T \end{aligned}$$



Specific Growth Rate for Weight (SGR-W, % change.day<sup>-1</sup>)

$$= \{[\ln(W2)-\ln(W1)]*100\}/T$$

L1 - length at T1, L2 - length at T2, W1 - weight at T1, W2 - weight at T2, T - time in days.

#### ***2.2.4 Pulse exposures***

On the day of exposure, water quality (pH, temperature, DO) were recorded, a sample was collected for subsequent ammonia analysis, and then the bulk of the organic wastes (80-90%) were siphoned from the tanks. When the normal water level had been restored, conditions were altered by diverting influent water, infusing industrial grade N<sub>2</sub> into the tanks and adding 2.5 L of ammonia stock solution (0.33 g.L<sup>-1</sup> of technical grade ammonia chloride in sand filtered seawater). Preliminary trials showed that this would achieve the desired UIA concentration.

All tanks had previously been adjusted to the same volume. Submersible aquarium pumps were placed in each tank immediately prior to altering the water quality to ensure that the conditions were uniform throughout the water column during the pulse exposure. Initially, large volumes of N<sub>2</sub> were infused to reduce the oxygen level rapidly; 60% saturation was achieved within 3 minutes. This concentration was subsequently maintained using small amounts of N<sub>2</sub> and air, infused through separate 50 mm airstones.

The return to ambient conditions was achieved by restoring influent water flow and removing the N<sub>2</sub>. The pumps were left running in the tank for one hour post-exposure to expedite the return to ambient conditions. Time-course sampling at the start of the trial showed that DO returned to ambient levels within 1 hour, and ammonia within 6 hours (data not shown). The pumps were placed in control tanks, and tanks not being exposed on a given week, as for the pulsed tanks, to reduce the possibility of any confounding influence on growth.

### 2.2.5 *Water quality*

DO, pH, ammonia and temperature for both pulse and challenge exposures were recorded before any alteration to conditions in the tank ( $T = 0$  h). pH and ammonia were then measured after 15-20 min ( $T = 15$  min), at 8 h (before restoring ambient conditions) and the morning following exposure ( $T = 24$  h). Once DO had stabilized, it was checked on average every 30-40 min and flow of  $N_2$  adjusted as required. DO, temperature and pH were recorded for all tanks every 2-3 days throughout the trial. Samples for ammonia analysis were taken from two tanks on each treatment not being pulsed each week to provide ambient ammonia levels. DO was measured using hand held meters (WTW Oxi96, TPS WP82-Y or Oxiguard Handy Gamma), which were calibrated daily and checked against saturated seawater and occasional Winkler titrations. The three meters gave similar readings when checked against each other. pH was recorded on a handheld TPS meter and probe (WP 81), calibrated daily in fresh buffers (phosphate at pH 7.00, borate at pH 9.28, after Brunos & Svoronos (1989)). Temperature was recorded using the thermistor on the DO meter, which was checked against a calibrated mercury thermometer.

All samples for ammonia analysis were collected in acid-washed glassware rinsed with de-ionized water. Samples were filtered through Whatman GF/C filters and frozen in polypropylene bottles (acid washed, rinsed in de-ionized water) for subsequent analysis. Total ammonia was measured within 4-5 weeks of the samples being collected, by the method of Solorzano (1968) but using the salicylate reagent of Bower & Holm-Hansen (1980). UA was calculated from pH and temperature using the equation in Bower & Bidwell (1978). Where necessary, samples were diluted with the sand-filtered seawater. Nitrite was analyzed by the diazotization method of Grasshoff (1989).

Influent flow rates were recorded every 5-10 days (mean  $\approx 1500 \text{ mL} \cdot \text{min}^{-1}$ ).

### ***2.2.6 Challenge exposure***

On day 45 of the growth trial, two tanks for each species on treatments 1, 2, 4 and 5 were exposed for 8 hours to more severe challenge conditions (Table 2.1, 2.4), as described above, but using a stock solution of  $1.33 \text{ g.L}^{-1}$  of ammonia chloride.

### ***2.2.7 Statistical analysis***

The raw data were tested for homogeneity of variance by examination of residual plots, generated by JMP 3.2 (SAS Institute). No transformations were required to meet the assumptions of ANOVA, as given in Underwood (1981). One-way ANOVA, using the replicate means for each treatment, was used to look for significant treatment effects, and Tukeys HSD was used to compare means.

## **2.3 Results**

### ***2.3.1 Behavioral observations***

Under ambient conditions, species-specific behavioral differences were observed. Blacklip abalone clustered in one or two spots in the tank, while greenlip abalone tended to gather in smaller groups dispersed more evenly over the available surfaces. Although generally inactive, some blacklip abalone were observed to be active during the day, while greenlip abalone were rarely observed to be active during the day. For both species, inactive animals had all tentacles withdrawn and shells clamped tightly onto the substrate. During the pulse exposure no change in behaviour was observed until the end of the exposure period, when signs of distress were evident (shells starting to lift off the substrate, tentacles becoming actively extended).

Although quantitative data on food consumption were not collected, ration was adjusted ad libitum for each tank. No difference in weight of food fed between treatments was observed. However, consumption tended to decline slightly the night following pulse exposure, but returned to normal the following night. Following the

challenge exposure, this reduction in consumption was even more marked, but again returned to normal the following night. Both species of abalone were also observed to be less active the night following the challenge exposure, with activity returning to normal the following night.

These effects were much more pronounced during the challenge exposure. In both species, the animals tended to separate from the clusters within 40 to 60 minutes of commencing the exposure, but once separated, they did not continue moving. Rather, in both species, the front of the shell was lifted off the substrate, and epipodial tentacles were extended. However, abalone in this position were not readily removed from the substrate, and if disturbed, clamped the shell back onto the substrate and withdrew the tentacles.

It was possible that the water current generated by the submersible pumps may have modified the response of the abalone to the stress induced by the pulses of poor quality water. In order to clarify this, a pulse exposure without the pumps was performed on the tanks in Treatment 3 at the end of the growth trial. No difference in behaviour was observed, compared to pulse exposures with pumps.

### ***2.3.2 Mortality***

Overall mortality was 0.8% for greenlip abalone and 2% for blacklip abalone during the growth trial. The higher figure for blacklip abalone was due to their tendency to crawl out of the tank. No mortality was observed as a direct result of the pulse or challenge exposures. There was no clinical evidence (such as lack of vigour, lesions, increased mortality, growth reductions) of either stress or microbial infection becoming established as a result of the pulse exposure regime. Two tanks of blacklip abalone did show signs of bacterial infection at different points during the growth trial, with some mortality, although no causative agent could be identified. The other replicate tank on this treatment showed no similar signs and these were considered anomalous events. These mortalities were excluded from the mortality calculations. Apart from these events, both species seemed generally healthy through out the growth trial.

### **2.3.3 Water quality**

Table 2.2 shows the ambient water quality for days when DO and ammonia were not manipulated. Table 2.3 shows the measured water quality achieved during pulses of ammonia and low DO. Table 2.4 shows the water quality achieved during the final challenge exposure. Nitrite was always undetectable. The initial and final data collected for each pulse (at T=0 h and T=24 h) were always consistent with ambient conditions and so were included in the ambient water quality in Table 2.2. Although it proved difficult to precisely control the ammonia level at the start of the pulse and challenge exposures, substantial increases in TA & UIA were achieved. Although total ammonia levels did not change greatly during the exposure period, the decline in pH reduced the UIA level.

One consequence of removing influent water during the exposure period was that temperature increased in line with the ambient air temperature, and pH decreased. However, these factors, while outside the control of this experimental system, further added to the reality of the simulation.

### **2.3.4 Growth data**

The growth data are shown in Figure 2.1. For greenlip abalone, growth rates ( $\approx 100 \mu\text{m.d}^{-1}$ ) were close to commercial growth rates. The blacklip abalone growth rates ( $\approx 15 \mu\text{m.d}^{-1}$ ) were approximately 10% of commercial growth rates. In terms of both length and weight, no significant difference was observed in growth of either species for any of the treatments ( $P > 0.05$ ). For blacklip abalone, Treatment 5 reduced growth, especially in terms of weight, but the degree of variation prevented the difference being significant.

## **2.4 Discussion**

The primary aim of this trial was to simulate a major systems failure, with an extended period of no influent water and little if any supplementary aeration. Establishing the alteration in water quality as rapidly as possible was intended to

exacerbate the impact of the simulation, as any changes in ammonia and oxygen from a real event would develop gradually rather than instantly. Although dissolved oxygen and ammonia were the primary water quality variables being manipulated, the lack of influent water flow also resulted in temperature and pH changes over 8 h. Changes in water temperature during the simulation reflected the ambient temperature, which was beyond experimental control, and the decline in pH was presumably due to respiration and metabolic excretions. Table 2.2 shows that substantially elevated levels of TA were achieved over the ambient level. While TA did not change greatly during the exposure period, the decline in pH did reduce the level of UIA. The tanks were cleaned prior to the interruption in water flow, so accumulated organic wastes would not have had any influence on water quality during the simulation.

It is considered possible that the water currents generated by the submersible pumps may have modified the response of the abalone to any stress resulting from the altered water quality during the intermittent exposures, perhaps by improving water movement past the gills. It is well known that abalone will orientate the shell to optimize the flow of water past the pores (Voltzow 1983; Tissot 1992). In order to determine what effect these factors may or may not have had in this trial, a pulse exposure was conducted at the end of the growth trial for Treatment 3 with out the submersible pumps. This trial showed no differences in behaviour from exposures with the pumps.

Based on observations of mortality and animal behaviour, the pulse exposures and exposure regime used in this trial did not result in more than a transient stress. Coupled with the lack of significant effects on growth, this indicates that the two species used in this trial are well able to withstand periodic declines in water quality. While a brief reduction in consumption was observed immediately following the pulse exposure, consumption returned to normal the following night. The abalone appeared healthy throughout the growth trial, with the exception of an isolated event, and mortality was similar to previous trials in this system (e.g. Maguire *et al.* 1996a,b). No clinical evidence of secondary complications such as significant

growth depression or bacterial infection were observed. Conditions in the final challenge exposure were apparently more stressful than the pulse exposures, based on observations of animal behaviour, but was still not sufficient to produce mortality within 3 days. Consumption and activity patterns returned to normal within 24 hours, as for the pulse exposures. The apparently transient nature of the stress from these exposures may have been partly due to the optimal quality of the ambient water (Table 2.2).

The lack of significant growth depression shows that any stress resulting from the pulse exposures was insufficient to affect growth for either species, in terms of length or weight, even when the stress was applied every 7 days. For blacklip abalone, growth was lower for Treatment 5 than in the other treatments in terms of both length and weight, which may indicate a biological effect on this species. The low overall growth in this species was presumably due to a combination of unsuitable diet and system design for this species, as animals from the same cohort under commercial conditions produced much better growth rates (R. Scharkie, Swansea, pers. comm.). This is further confirmed by the observation that once the experimental abalone were returned to a commercial grow-out system, they rapidly attained market size (R. Scharkie, Swansea, pers. comm.). The lack of growth in this species does suggest that these animals were already under some form of stress, and thus more likely to be affected by the pulse or challenge exposures.

Harris *et al.* (1997, 1998a, 1999a) reported that greenlip abalone were more sensitive than fish and other invertebrate species when chronically exposed to elevated UIA, nitrite or DO levels below saturation. For DO and UIA, growth declined through the experimental range in these chronic studies (25-188  $\mu\text{g UIA.L}^{-1}$  and 8.9-4.2  $\text{mg DO.L}^{-1}$  (117-55% saturation)). These levels of UIA and DO were achieved in the pulse exposure without producing a significant growth depression. The pH after 8 h of either challenge or pulse exposure was in the range 7.91-7.62. Harris *et al.* (1999b) found that chronic exposure to pH 7.76 significantly reduced growth in both species of abalone compared to controls at pH 8.27. The

temperatures recorded after the 8 h of either pulse or challenge exposures were still within the tolerance limits defined by Gilroy & Edwards (1998) for these species.

Harris *et al.* (1998b, 1999b) reported some histopathological changes in gill and kidney tissue from exposure to the levels of UIA, DO and pH achieved in the pulse exposures of this study. While no samples were taken for histological analysis, the lack of growth depression, except possibly for the blacklip abalone on Treatment 5, would suggest that no similar changes would have been found in this study. Harris *et al.* (1998a, 1999a) reported significant growth depression under those conditions resulting in histopathological effects.

The chronic exposures outlined above were based on continuous exposure to altered water quality for several weeks. The pulse and challenge exposures in the current trial were for 8 h, during the normally inactive daytime period. It is likely that gill activity is reduced during this quiescent period. Certainly for molluscs in general this is the case with activity levels, heart rate, gill ventilation and gill perfusion rates and resulting oxygen uptake intimately related. This has been shown for sedentary species such as the blue mussel (*Mytilus edulis*) (Bayne 1971) and more active gastropods with a range of survival and feeding strategies (Morton 1990). For abalone, external factors such as temperature and oxygen tension have been clearly linked to factors such as heart rate, blood pressure and resulting oxygen uptake (Nimura & Yamakawa 1989; Russell & Evans 1989). In the quiescent period, tissue exposure to toxicants such as ammonia may be reduced as a result of lower gill perfusion rates and therefore reduced branchial uptake and tissue perfusion rates. Low DO has been shown to reduce heart rate in several abalone species (Nakanishi 1978; Russell & Evans 1989), and this may have provided some protection for internal tissue in this trial. It is possible that a similar mechanism may work in crustaceans, as Allan & Maguire (1991) report that tiger prawns (*Penaeus monodon*) tolerated conditions for several hours that resulted in significant growth reductions from a more extended exposure over several weeks.

Studies of haliotids in the wild shows that these animals tend to be found in large groups, commonly stacked around boulders and in crevices at various depths



(Poore 1972; Shepherd 1973; Shepherd & Partington 1995). Under such conditions, periods of low DO and elevated ammonia concentrations would not be unexpected, particularly in warmer weather and low water exchange, or when decaying organic matter accumulates (Wells *et al.* 1998a). While no references could be found on water quality in such aggregations under these conditions, the available literature indicates that gastropod molluscs are physiologically and biochemically well suited to cope with episodes of environmental hypoxia (Brix *et al.* 1979; Gäde & Ellington 1983; Storey & Storey 1990). It is also well documented that gastropods including abalone are biochemically and physiologically adapted cope with the functional hypoxia which develops in the pedal muscle, which would also enable them to deal with environmental hypoxia (Gäde & Grieshaber 1986; Gäde 1988; Wells *et al.* 1998a,b). The tolerance to hypoxia shown in this trial is therefore not unexpected. The growth data and other observations also show a tolerance of acute elevations in environmental ammonia levels, at least when combined with hypoxia.

It is possible that a significant impact on growth may have been observed if the pulse exposures had been conducted during the nocturnal foraging period, or over a longer period (eg 12-24 h). Potentially, the altered water quality would then have had a more direct effect on the metabolic processes of the abalone. Abiotic factors such as stocking density (Chapter 5, 6), nocturnal activity and/or accumulating organic wastes (Chapter 7, 8) may contribute to alterations in water quality which may impact on growth in this manner.

While it is clear that the exposure conditions in this trial were well within the tolerance of greenlip and blacklip abalone on an intermittent basis, chronic exposure to elevated environmental ammonia in conjunction with low DO levels may exceed the biological limit of tolerance, resulting in a degree of physiological disturbance which may be sufficient to reduce somatic growth and hence growth rates. The effects of such exposures are investigated in Chapters 3 & 4.

## **2.5 Conclusion**

The results indicate that the two abalone species were remarkably resilient to periodic short-term exposure to poor water quality that will significantly reduce growth if present chronically as individual exposures. The exposure conditions, either as a periodic pulse or more severe challenge, did not result in mortality above acceptable background levels. Transient (less than 24 h) effects on consumption and foraging behaviour were observed, especially after a more severe challenge exposure at the end of the growth trial. Based on lack of mortality and behavioral observations, exposure history had little if any effect on the response to a more severe challenge exposure.

Treatment	Day 1	7	14	21	28	35	42	45	47
1							●		
2	■						●		
3	■			■					
4	■		■		■		●		
5	■	■	■	■	■	■	●		

Table 2.1 Pulse exposure treatments for blacklip and greenlip abalone. Between exposures, abalone were held at ambient water quality (Table 2.2). At the intervals indicated above, water quality was altered as described in Table 2.3 for 8h. ■= Pulse exposure. ● = Challenge exposure. All abalone were individually tagged, then weighed and measured 2-3 days before the first exposure on Day 1, and again on Day 47. Treatment 1 is the control.

Treatment	Dissolved Oxygen (mg.L <sup>-1</sup> )	pH	Temperature (°C)	TA (mg.L <sup>-1</sup> )	UIA (µg.L <sup>-1</sup> )
Greenlip					
1	7.52±0.02	8.09±0.00	17.19±0.05	0.04±0.04	1.40±1.15
2	7.56±0.01	8.12±0.01	17.25±0.04	0.01±0.02	0.44±0.55
3	7.58±0.00	8.11±0.00	17.13±0.01	0.046±0.5	1.54±0.47
4	7.57±0.01	8.11±0.00	17.32±0.01	0.06±0.01	1.98±0.23
5	7.50±0.01	8.11±0.00	17.45±0.45	0.03±0.01	1.07±0.22
Blacklip					
1	7.48±0.02	8.10±0.00	17.19±0.05	0.05±0.03	1.78±1.16
2	7.57±0.01	8.12±0.00	17.17±0.00	0.04±0.01	1.33±0.45
3	7.56±0.01	8.11±0.00	17.16±0.02	0.05±0.01	1.58±0.20
4	7.61±0.01	8.13±0.00	17.32±0.02	0.06±0.01	1.95±0.31
5	7.47±0.02	8.11±0.00	17.43±0.04	0.05±0.01	1.61±0.23

Table 2.2 Ambient water quality during growth trial, based on weekly samples. Due to lack of difference from ambient conditions, data at T=0 h and T=24 h from the pulse exposures are included in this data (mean±SE, n=3).

Treatment	DO (mg.L <sup>-1</sup> )	pH	Temp.(°C)	TA (mg.L <sup>-1</sup> )		UIA (µg.L <sup>-1</sup> )	
		T = 8 h	T = 8 h	T = 15 min	T = 8 h	T = 15 min	T = 8 h
Greenlip							
2	4.18±0.13	7.62±0.06	22.23±0.35	5.56±0.26	5.15±0.51	182.73±5.50	87.07±26.86
3	4.47±0.03	7.81±0.01	18.73±0.03	5.08±0.08	3.82±0.26	178.41±1.81	71.52±8.64
4	4.35±0.01	7.72±0.03	19.60±0.05	3.81±0.03	3.83±0.04	108.43±1.39	59.90±3.28
5	4.34±0.05	7.67±0.02	20.61±0.46	5.66±0.04	5.27±0.08	160.77±0.62	74.29±2.22
Blacklip							
2	4.33±0.12	7.75±0.09	21.97±0.18	5.32±0.38	5.95±0.24	183.10±13.34	120.16±28.23
3	4.52±0.05	7.91±0.01	18.95±0.05	5.41±0.18	3.62±0.11	187.45±3.70	87.27±4.57
4	4.44±0.03	7.82±0.04	19.52±0.11	4.12±0.20	3.97±0.11	121.50±7.84	78.47±5.33
5	4.37±0.05	7.72±0.03	20.66±0.53	5.57±0.17	5.21±0.15	161.11±6.51	82.95±1.15

Table 2.3 Average conditions during pulse exposures. DO values are based on readings taken ≈30–40 minutes apart during the 8h exposure period (± SD); pH, temperature and ammonia data are means from replicate tanks (n=3, ± SE). Water quality data taken before the exposure period began (T=0 h) and the day following the exposure (T=24 h) are included in Table 2.1, as they did not differ from ambient conditions. pH and temperature at T=15 min did not differ from pH at T=0 h.

Treatment	Greenlips	Blacklips
DO (mg.L <sup>-1</sup> )	2.36±0.10	2.35±0.07
pH (T = 8 h)	7.73±0.035	7.85±0.02
Temp.(°C, T = 8 h)	19.62±0.77	19.61±0.77
TA (mg.L <sup>-1</sup> ), T = 15 min)	19.14±4.24	16.50±2.81
TA (mg.L <sup>-1</sup> ), T = 8 h)	15.53±3.60	16.30±3.20
UIA (µg.L <sup>-1</sup> ), T = 15 min)	625.77±144.10	547.87±95.86
UIA (µg.L <sup>-1</sup> ), T = 8 h)	384.47±115.23	372.43±107.98

Table 2.3 Treatment means of conditions during challenge exposure (mean±SE, n=4). DO values are based on readings taken ≈30-40 minutes. pH, temperature and ammonia data were based on single readings taken as indicated, from replicate tanks on each treatment.

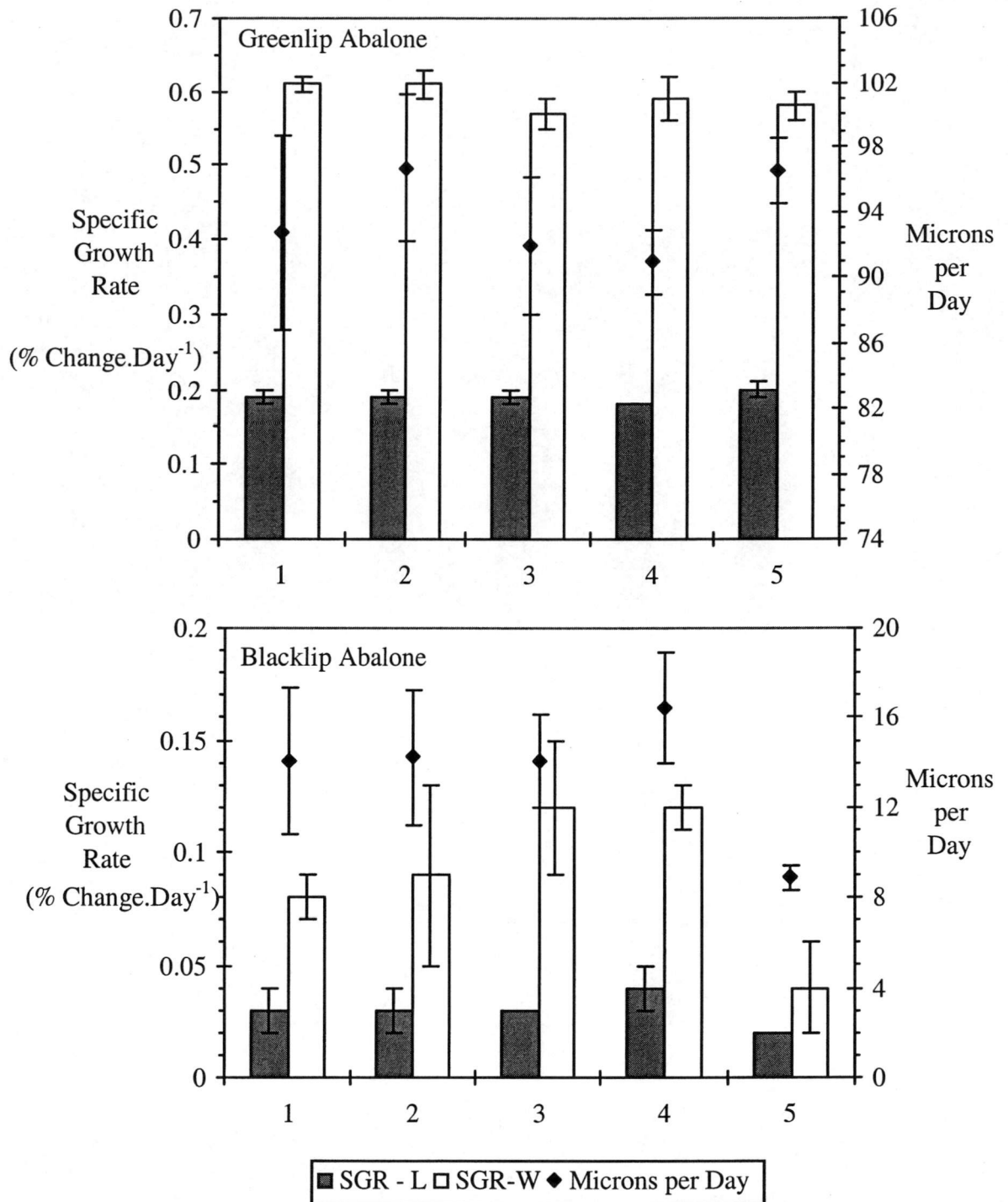


Figure 2.1 Growth rate data from pulse exposures of two species of abalone (mean±SE, n=3). Treatments are given in Table 2.1. SGR-L = Specific growth rate for length (% increase in length per day), SGR-W = specific growth rate for weight (% increase in live bodyweight per day)

## **CHAPTER 3**

### **Chronic Exposure To Low Dissolved Oxygen Levels Combined With Elevated Environmental Ammonia Concentrations**

#### **3.1 Introduction**

Somatic growth is the summation of a diverse range of cellular and biochemical processes, with maximum growth occurring under optimal environmental conditions. The interaction of biotic and abiotic factors in the environment will affect these processes to an extent dependent on the nature of the factor and duration of the exposure. One consequence of the high densities used in intensive aquaculture systems is the potential for degradation of water quality, with factors such as elevated environmental ammonia concentrations and low dissolved oxygen levels potentially reducing growth rates and directly compromising stock health. Such alterations in water quality are also likely to exert a sub-lethal stress which may predispose the stock to secondary infections which will further reduce growth rates or increase mortality.

In the marine environment ammonia is normally present only at low levels, and organisms such as abalone are not normally required to deal with elevated environmental levels. Hypoxic conditions of both physiological and environmental origin are commonly encountered by a range of invertebrate species including abalone, and these species have developed the necessary biochemical machinery to provide energy for metabolic processes during hypoxic episodes (Gäde & Ellington 1983; Wells *et al.* 1998a). In order to optimize the environment for the species being cultured, the aquaculturist needs to know how factors such as ammonia and oxygen will interact to affect growth and health.

It is clear from the literature that when both vertebrate and invertebrate marine organisms are exposed to environmental ammonia, plasma ammonia levels initially



increase (Wilson & Taylor 1992; Chen & Cheng 1993; Chen *et al.* 1994; Knoph & Thorud 1996; Schmitt & Uglow 1997). Excess internal ammonia can be actively excreted against this concentration gradient and ammonia levels will stabilize at a point dependent on such factors as the capacity of the active excretion mechanism, severity of the exposure conditions and duration of the exposure (Schmitt & Uglow 1997; Schmitt & Santos 1999). In teleosts, environmental ammonia exposure is known to disrupt various components of glycolysis (Sousa & Meade 1977; Arillo *et al.* 1981; Dabrowska & Wlasow 1986; Begum 1987; Jeney *et al.* 1992; Vedel *et al.* 1998). Since molluscs are known to utilize the same basic glycolytic pathways (Hochachka *et al.* 1983; Livingstone & De Zwaan 1983), it is likely that ammonia exposure will exert a similar effect, and has been demonstrated in fresh water mussels (*Lamellidans marginalis*) (Chetty & Indira 1995). Phosphofructokinase, one of the enzymes important in regulating glycolysis in both vertebrate (Sousa & Meade 1977) and invertebrate (Hochachka *et al.* 1983) pathways, is directly stimulated by ammonia and thus elevated serum ammonia levels may artificially increase the rate of glycolysis, leading to a depletion of the substrates which in time will reduce the rate of glycolysis. It is not clear whether this is an incidental effect of ammonia exposure, or designed to meet the additional energetic cost of exposure to elevated environmental ammonia levels.

Osmoregulation is known to be an energy intensive process under normal conditions (Spaargaren 1990; Lucu & Pavicic 1995), and disturbances in osmoregulation as a result of exposure to ammonia will add to this demand and divert energy from tissue growth (Knoph & Thorud 1996; Schmitt & Santos 1999).

There is an extensive volume of literature on the effects of ammonia or low dissolved oxygen as individual variables. Various authors have also investigated the effects of elevated environmental ammonia levels in conjunction with low DO on both vertebrate (Downing & Merckens 1955; Merckens & Downing 1957; Lloyd 1961; Thurston *et al.* 1981c; Alabaster *et al.* 1983; Wajsbrodt *et al.* 1991; Tudor *et al.* 1994) and invertebrate (Allan *et al.* 1990; Wajsbrodt *et al.* 1990; Chen & Nan 1992a) species. These studies were based on acute exposures, mostly using 96 hr LC<sub>50</sub> values as a

measure of toxicity, and demonstrated that DO levels below 85-55% saturation increased the toxicity of ammonia. These studies did not measure any effect of longer term exposures (over several weeks) on growth or survival.

While it is well known that abalone can tolerate both functional and environmental hypoxia (Gäde 1988; Wells *et al.* 1998a), Harris *et al.* (1999a) showed that this tolerance is finite with growth reductions resulting from chronic exposure to oxygen levels less than 80% saturation. Elevated environmental ammonia levels are also known to cause growth depression in abalone (Harris *et al.* 1998a; Basuyaux & Matthieu 1999). While it is clear from Chapter 2 that blacklip and greenlip abalone can tolerate intermittent exposure to elevated ammonia levels in conjunction with low DO levels, the chronic effects of such combined exposures are not known. In the present study a growth trial was conducted which held greenlip and blacklip abalone for up to 67 days at two different levels of dissolved oxygen combined with different levels of environmental ammonia. Significant growth reductions were observed as a result of the experimental exposures, with evidence that low dissolved oxygen alleviated the effect of environmental ammonia.

## 3.2 Methods & Materials

### 3.2.1 Bioassay System

The trial was conducted at a research facility at Bicheno, Tasmania, Australia (41°5'S, 148°18'E). The system was designed to supply oceanic, sand-filtered seawater (drawn from rocky, exposed coastline, salinity 34-35 g.L<sup>-1</sup>) by gravity from individual reservoirs to each of three experimental bioassay units per treatment, via a constant head column (1.8m \* 150 mm PVC pipe), which was fitted with a float valve to ensure a constant flow-rate through the units as the level in the reservoirs decreased. The reservoirs were drained and re-filled with fresh seawater daily. A common length (3.5 m) of 4 mm black poly-propylene tubing was used to supply the individual units from the bottom of the constant head column to standardize the flow rates. Average water flow through the units, measured at the outlet, ranged from 180 to 230 mL.min<sup>-1</sup>. Dissolved oxygen levels were adjusted by combining industrial grade nitrogen and

oxygen (>99.5 % pure) in a mixing chamber and infusing this mixture into the bottom of the constant head column just above the water outlet. Influent water entered the column at the top, maximizing the time for equilibration with the infused gas mixture. Separate mixing chambers were used for the two experimental DO levels. Ammonia was adjusted by adding the required amount of technical grade  $\text{NH}_4\text{Cl}$  to the reservoirs as they were refilled.

The 70 L bioassay units were made of fibreglass, based on a double cone design. Water entered through an inlet in the side of the bottom cone (approx. 30 cm from the bottom of the cone), and exited through the apex of the top cone, with access for cleaning, feeding and water sampling via a threaded access port in the top cone. The greenlip and blacklip abalone were held in separate cylindrical cages (35 cm \* 100 mm PVC pipe, with polypropylene mesh walls and ends) suspended in the water column. The units were cleaned every 7-10 days by placing cages in a replicate unit on the same treatment, while the destocked unit was drained, scrubbed, hosed with fresh water, and then refilled from the appropriate header column. The cages were fully submerged when replaced in the clean bioassay unit. By sequentially cleaning one triplicate tank for each treatment, disturbance to the pre-set DO level was minimized. A valve in the bottom of the lower cone was opened daily to remove accumulated particulate wastes. The 4 mm tubing connecting units to the header column was replaced with clean tubing (re-used tubing which had been scrubbed internally, flushed with hot fresh water and dried) every 15 to 20 days, and fresh food was placed in the cages every 2-3 days. Ration was adjusted on a demand basis for each tank and for each species. Heaters were placed in the constant head columns to control temperature, and small submersible aquarium pumps were placed in each bioassay unit to provide water movement and circular water currents to maximise collection of solids in the bottom cone.

### ***3.2.2 Experimental Abalone***

The blacklip abalone were obtained from commercial stock at Swansea, Tasmania, Australia, and were 18-24 months old at the start of the trial (mean initial

length =  $37.1 \pm 3.0$  mm, mean initial weight =  $8.37 \pm 2.14$  g,  $n=360$ ). The greenlip abalone were obtained from commercial stock at Bicheno, Tasmania, Australia and were 24-30 months old at the start of the trial (mean initial length =  $41.8 \pm 3.0$  mm, mean initial weight =  $9.28 \pm 1.94$  g,  $n=360$ , means  $\pm$  SD). Both species had been held in 270 L holding tanks for 4-6 weeks before being used in the current trial.

A spatula was used to remove abalone from the holding tanks for allocation, in groups of 5, to the bioassay units. Abalone were weighed (to 0.01 g) and measured (to 0.1 mm, using Vernier calipers). Polyethylene tags (Hallprint, Adelaide, Australia) had previously been applied, and any missing tags were replaced using cyano-acrylate adhesive gel. The same diet, a commercial formulation (ABCHOW) modified by the addition of 10% algal meal, was used prior to and during the trial for all abalone. Both species were initially stocked at 20 animals per cage.

### 3.2.3 Growth Trial

Once weighed, measured and allocated the abalone were acclimatized to the system for 3-4 days before commencing ammonia exposure ( $T=0$ ). The experimental design was based on one control treatment, and 5 experimental treatments. The 3 exposure levels of ammonia (nominally 1.3, 2.1 and 31.8 mg total ammonia (TA).L<sup>-1</sup>, designated as low – LA, medium – MA and high – HA respectively) were sequentially achieved by starting with the lowest level on Day 1, and gradually increasing the concentration of ammonia in the higher treatments over several days. Reductions in DO were commenced 5-6 days after ammonia exposure commenced (nominally 60 % (low - LO) and 80% (medium - MO) saturation). The five experimental combinations of ammonia and low DO used for the growth trial are shown in Table 3.1. A control treatment, nominally at 100% saturation with no added ammonia, was also used. While the control units were aerated, the experimental units were not.

The trial was conducted from February to April 25, 1999 (67 days in total, with Day 1 being the first day ammonia was added to the reservoirs). By Day 17, substantial mortalities had occurred in MO/HA, and the nominal ammonia level was reduced to 24.5 mg TA.L<sup>-1</sup> for this treatment. At the same time, the stocking density for all experimental treatments and the control was reduced to 15 animals per cage.

Due to excessive mortality in MO/HA up to this point, 3 abalone from the MO/MA exposure were used to replace some animals in one replicate from the MO/HA treatment. Otherwise replacement animals, when required, were taken from the other replicates of the same treatment. Length and weights were not re-recorded at this time; data recorded at the start of the trial was used to calculate the growth rates at the end of the trial for these replacement abalone.

Growth data were calculated from the change in length and weight of individual abalone from  $T = 0$ .

The following equations were used to calculate growth rates (as % change per day):

$$\begin{aligned} \text{Specific Growth Rate for Length (SGR-L, \% change.day}^{-1}\text{)} \\ = \{[\ln(L1) - \ln(L0)] * 100\} / T \end{aligned}$$

$$\begin{aligned} \text{Specific Growth Rate for Weight (SGR-W, \% change.day}^{-1}\text{)} \\ = \{[\ln(W1) - \ln(W0)] * 100\} / T \end{aligned}$$

$L0$  - length at  $T=0$  (in mm),  $L1$  - length at  $T=1$  (in mm),  $W0$  - weight at  $T=0$  (in g),  $W1$  - weight at  $T=1$  (in g),  $T$  - time from  $T0$  to  $T1$  in days.

Length:weight ratios were calculated by dividing the length of individual abalone by the live weight of that abalone for both the beginning ( $L0:W0$ ) and the end of the trial ( $L1:W1$ )

### 3.2.4 Water Quality

DO was recorded at least once per day, and normally twice (morning and afternoon), using hand held meters (WTW Oxi96, TPS WP-82Y or Oxiguard Handy Gamma), which were calibrated daily using saturated seawater and checked against regular Winkler titrations. pH was recorded daily using a hand held TPS unit and probe (WP 81), calibrated daily in fresh buffers (phosphate at pH 7, borate at pH 9.28, after Bruno & Svoronos 1989). Temperature was recorded using the thermistor on the DO meter, which was checked against a calibrated mercury thermometer.

Samples for ammonia analysis were collected daily from at least one and usually two replicate tanks on each treatment. Every 5-7 days, samples were taken from all

tanks. All samples for ammonia analysis were collected in acid-washed glassware that had been rinsed with de-ionised water. Samples were filtered through Whatman GF/C filters, and frozen in polypropylene bottles (acid washed, rinsed in de-ionised water) for subsequent analysis. Total ammonia (TA) was measured on thawed samples using the basic method of Bower & Holm-Hansen (1980), but replacing the alkaline and oxidizing reagents with those described in Solorzano (1969), mostly within 3-8 weeks of collection. Un-ionised ammonia (UIA) was calculated from the TA, temperature and pH of the original sample using the equation in Bower & Bidwell (1978). Ambient seawater from the same source used to fill the reservoirs was used for making all standards and diluting thawed samples such that the TA was under  $0.5 \text{ mg TA.L}^{-1}$ . Nitrite samples were collected from all replicate tanks every 7-10 days, filtered and frozen for subsequent analysis as described above. Thawed samples were analyzed for nitrite using the diazotisation method (Grasshoff 1989).

### *3.2.5 Statistical Analysis*

Analysis of water quality data was based on replicate tank means, averaged for the whole trial. It was assumed that because of the low stocking density and with the animals being fed to excess that there were no significant interactions between individual abalone, and therefore results for individual abalone in each treatment were used for statistical analysis with replicate nested within treatment to allow for any variation between replicate tanks.

All data were checked for heterogeneity of variance by observation of residual plots generated by JMP 3.2. No transformations were required for either water quality or growth data, in order to meet the criteria given in Underwood (1981) for performing ANOVA. ANOVA was used to look for significant treatment effects, and Tukeys HSD was used to determine which means were significantly different. Water quality was analysed using JMP 3.2 (SAS Institute), and SPSS 10.02 (SPSS Inc.) was used to analyse the growth data.

### 3.3 Results

#### 3.3.1 Water Quality

Table 3.1 shows the mean total ammonia (TA), un-ionised ammonia (UIA) and oxygen data measured throughout the trial, with data from the statistical analysis given in Table 3.2 (n, F ratio, degrees of freedom, P value). Oxygen levels in MO and LO treatments differed significantly from each other, and were significantly lower than the control (78.012, 5, 10,  $P < 0.001$ ). While there was some variation in TA levels, significantly different levels were maintained between LA, MA and HA treatments (738, 5, 10,  $P < 0.001$ ). A degree of nitrification is evident from the nitrite levels in Table 3.1, and there was also a slight decline in pH from ambient (8.1).

#### 3.3.2 Mortality

Because there was a degree of tag loss through the trial, not all the original abalone could be re-measured at the end of the trial. Tag loss ranged from 10-25% for individual tanks. There was 30-40% mortality in the MO/HA treatment by Day 17, in both greenlip and blacklip abalone. On Day 18, the nominal ammonia level was reduced to  $31.8 \text{ mg TA.L}^{-1}$  for this treatment. Furthermore, the gas delivery system was modified to make the DO saturation less variable, and the stocking density was reduced to 15 abalone per cage for all treatments. On Day 25, there was a mortality event in one replicate unit on each of the LO treatments (LO/LA and LO/MA), and it was decided to remove these replicates from the growth trial, leaving duplicate rather than triplicate units on these treatments. Between Day 17-51, mortality was less than 2.2% for either species on any treatment (except for the losses noted on Day 25).

An hypoxic stress event occurred on Day 51, when all the bioassay units on 4 treatments (control, MO/LA, MO/MA and MO/HA) received no influent water for a period of 20 h due to a technical error. Water quality in the bioassay units at the time this was discovered and the subsequent mortalities are shown in Table 3.3. While this resulted in substantial mortality in the experimental treatments, especially among the greenlip abalone, there were sufficient survivors to enable at least two replicate tanks to be maintained for the remainder of the growth trial. Un-tagged animals were used

where necessary to maintain the biomass loading, but were not used in calculating the growth data. The control treatment being aerated prevented the substantial decline in oxygen levels seen in the experimental treatments.

It can be seen from Table 3.3 that for the greenlip abalone in the MO/LA and MO/HA treatment, there was considerable variation between one replicate and the other two replicates in terms of mortality. Examination of the water quality data for the individual tanks leading up to the hypoxic event does not show any differences in terms of background water quality which may explain these results. This variation precluded statistical analysis of the mortality data.

### ***3.3.3 Growth data***

The growth data, based on length and weight data collected from the tagged survivor, are shown in Figures 3.1 & 3.2. Length data from the mortalities incurred on Day 51 was also included in the growth calculations, but not the weight data. Data on the statistical analyses are shown in Table 3.2 (F ratio, degrees of freedom and P values).

For both species the specific growth rate for weight (SGR-W) at low saturation (LO) was significantly better than for the same animals at the same ammonia exposure category at moderate (MO) saturation (blacklip – 15.158, 5, 175,  $P < 0.001$ ; greenlip – 30.926, 5, 141,  $P < 0.001$ ). This was despite the LOMA treatment having a significantly higher concentration of ammonia than the MOMA treatment ( $2.48 \pm 1.08$  vs  $1.88 \pm 0.09$  mg L<sup>-1</sup>, Table 3.1). Differences in the specific growth rate for length (SGR-L) were not as clear as for SGR-W. There was a general pattern of growth evident for both species in which the lowest growth rates occurred in the MO/MA and MO/HA treatments.

Blacklip abalone had much lower overall growth rates than greenlip abalone, and showed weight loss in two treatments (MO/MA & MO/HA). In terms of SGR-W, growth in the MO/MA and MO/HA treatments was significantly lower than in the control, LO/LA and LO/MA treatments (Figure 3.1). For SGR-L, only the MO/HA and LO/MA treatments were significantly lower than control (5.722, 5, 189,  $P < 0.001$ ) (Figure 3.1).



For greenlip abalone (Figure 3.2), the lowest growth in terms of SGR-W occurred in the MO/MA and MO/HA treatments, being significantly lower than in the control and the other three experimental treatments. Growth in the LO/LA and LO/MA treatments was significantly higher than for the control and MO/LA treatment. In terms of SGR-L, only the MO/LA treatment was not significantly lower than the control (20.224, 5, 186,  $P < 0.001$ ). Growth in the MO/HA treatment was significantly lower than in LO/MA treatment, but not different from growth in the MO/MA and LO/LA treatments.

Length:weight ratios did not differ between treatments for either species at the start of the growth trial (L0:W0), or for blacklip abalone after the growth trial (L1:W1) (data not shown). However, for greenlip abalone at the end of the growth trial, the ratio was significantly higher in the MO/MA and MO/HA treatments than in the LO/LA and LO/MA treatments (5.074, 5, 141,  $P < 0.001$ ). Length:weight ratios for the control and MO/LA treatments did not differ from the other treatments.

### 3.4 Discussion

Traditionally, the toxic effects of ammonia exposure have been primarily attributed to UIA due to the known lipid solubility of small neutral molecules such as  $\text{NH}_3$  and implied ease of transfer across lipid rich cell membranes, plus the fact that this form predominates in most aquatic environments (Bower & Bidwell 1978; Wilkie 1997). However, there is increasing evidence that the ionized form ( $\text{NH}_4^+$ ) may have more effect in the marine environment due to the greater cation permeability of branchial tissue in most marine species (Evans & Cameron 1989; Wilson & Taylor 1992; Wilkie 1997). The results from the current trial are therefore presented and discussed in terms of TA rather than UIA.

Except for an isolated event, mortality over the bulk of the growth trial (Day 17-51) was generally low. After some initial losses, particularly in the MO/HA treatment, subsequent mortality was controlled by decreasing the highest ammonia level and reducing the stocking density in all treatments mortality virtually ceased. The cessation of mortality once the ammonia concentration was reduced may have been

due, at least in part, to the abalone acclimating to ammonia exposure. In a similar trial investigating ammonia as a single variable, Harris *et al.* (1998) also had to reduce the highest experimental ammonia level (from 363 to 159  $\mu\text{g.L}^{-1}$  UIA) to stop initial mortality.

Significantly higher mortality in greenlip abalone relative to the control during chronic bioassays either from elevated ammonia (45% at 159  $\mu\text{g UIA.L}^{-1}$ ) or from low DO levels (62% mortality at 63% saturation) as individual exposures was reported by Harris *et al.* (1998a) and Harris *et al.* (1999a) respectively. In the current trial, using treatments that combined these conditions, significant mortality was not evident for the bulk of the growth trial (34 days) in the absence of other external events. This suggests that abalone may be able to better survive when both stresses are combined rather than when they occur separately.

Some of the experimental exposure conditions achieved in this trial resulted in significant growth depression relative to the control for both species. The growth rates of control abalone were low compared to reported growth rates for cultured abalone (80-100  $\mu\text{m.d}^{-1}$ ) (Aviles & Shepherd 1996; Fleming, van Barneveld & Hone 1996; Fleming & Hone 1996), but similar to growth rates from other bioassay trials with greenlip and blacklip abalone in this system (Harris *et al.* 1998, 1999a,b). The possible reasons for the observed reductions in growth are discussed below.

The nitrite levels observed in this trial (Table 3.1) were found to significantly depress growth in greenlip abalone during chronic exposure (82 days) (Harris *et al.* 1997). Since the nitrite levels in the current trial increased over time (data not shown), these high levels were not present throughout the whole trial. Due to similar growth rates being observed from the control and MO/LA treatments for both species, despite the presence of 0.93 mg  $\text{NO}_2 \cdot \text{L}^{-1}$  in the MO/LA treatment, it was considered that nitrite was not elevated long enough to significantly affect growth. It is also possible that elevated nitrite levels in conjunction with low DO may not have the same impact as nitrite alone. While pH was lower than the ambient level of 8.1, the levels shown in Table 3.1 are still above those reported by Harris *et al.* (1999b) as reducing growth in either blacklip or greenlip abalone. Thus, while both pH and nitrite obviously have the

potential to cause growth depression in abalone, it was considered that the predominant effect on growth in the current study was from experimental exposure conditions.

There are some data on exposure of abalone to either low DO or elevated environmental ammonia. Basuyaux & Matthieu (1999) found growth in *H. tuberculata* was significantly reduced in terms of length and weight after a 14-day exposure to 10 mg TA.L<sup>-1</sup>. Harris *et al.* (1998a) reported significant growth depression in greenlip abalone at 2.65 mg TA.L<sup>-1</sup> (54 µg UIA.L<sup>-1</sup>) in terms of length and 6.16 mg TA.L<sup>-1</sup> (110 µg UIA.L<sup>-1</sup>) in terms of weight, with growth declining throughout the entire experimental range (1.01-9.04 mg TA.L<sup>-1</sup>, 25-188 µg UIA.L<sup>-1</sup>). For DO, Harris *et al.* (1999a) found growth of greenlip abalone was significantly lower than controls at 73% saturation (5.6 mg DO.L<sup>-1</sup>) and 81% (6.2 mg DO.L<sup>-1</sup>) for length and weight respectively. These authors also found that while SGR-L declined over the entire range (100-55 %, 7.7-4.2 mg DO.L<sup>-1</sup>), depression in terms of SGR-W did not occur until oxygen saturation was lowered to 81%. Harris *et al.* (1999a) also found that chronic exposure to 4.2 mg DO.L<sup>-1</sup> (55% saturation) resulted in a negative growth rate in terms of weight for greenlip abalone, as seen in the MO/MA and MO/HA treatments in the current study for blacklip abalone. The fact that the negative growth rates reported by Harris *et al.* (1999a) occurred at lower oxygen levels may be due to a species difference, or to an interaction between environmental ammonia exposure and low oxygen levels. Combining 56% DO with 2.48 mg TA.L<sup>-1</sup> (LO/MA) showed no significant difference in SGR-W for blacklip abalone and a significant increase in SGR-W for greenlip abalone compared to the controls. This indicates that an interaction is occurring in the effects of low DO and high ammonia to modify the physiological response to these modified environments.

For greenlip abalone, growth in terms of weight was more sensitive to the exposure conditions than growth in terms of length (L1:W1 was greater for MO/MA and MO/HA on the one hand than for LO/LA or LO/MA on the other). This suggests that either shell growth was continued at the expense of somatic growth, the combined exposure conditions had more effect on somatic growth than shell growth or that shell

and somatic growth are not directly linked. This is supported by the study of Palmer (1981), who showed that shell growth in marine gastropods is not directly linked to somatic growth and can proceed during starvation or periods of low energy intake.

Significant growth reductions in the current trial (in MO/LA and MO/MA treatments) occurred at environmental ammonia levels lower than would be expected based on the data given in Harris *et al.* (1998) for abalone exposed to ammonia as an individual variable. The oxygen levels that produced significantly lower growth rates than the control were higher than those reported by Harris *et al.* (1999a) as causing significant growth reduction in greenlip abalone. These findings indicate that low concentrations of environmental ammonia combined with modest declines in dissolved oxygen have a greater impact than the individual variables, a conclusion which has also been reported for penaeids (Allan *et al.* 1990; Wajsbrot *et al.* 1990; Chen & Nan 1992), salmonids (Downing & Merckens 1955; Lloyd 1961; Thurston *et al.* 1981; Alabaster *et al.* 1983) and a range of non-salmonid fish species (Merckens & Downing 1957; Wajsbrot *et al.* 1991; Tudor *et al.* 1994).

Chronic exposure to either environmental ammonia or hypoxia is known to reduce food consumption in abalone (Harris *et al.* 1998a, 1999a; Basuyaux & Matthieu 1999). While not quantified in this trial, the measured ration, adjusted on demand for each bioassay unit, was similar for MO/LA, MO/MA, LO/LA and LO/MA treatments and were all lower than the control. The lowest ration was fed to the MO/HA treatment. Growth rate depression in terms of weight would be the result of energy expenditure exceeding the energy available from the ingested ration. If the differential between ingested energy and energy expenditure is maintained for long enough then muscle tissue will be catabolised to make up the difference. Muscle loss has been reported in prawns exposed to hypoxia (Rosas *et al.* 1999), and in abalone during extended starvation (Segawa 1991). This would explain the negative growth observed for blacklip abalone in the current study, and has also been reported for greenlip abalone exposed to hypoxia (Harris *et al.* 1999a).

It can also be seen from the growth data for both species that SGR-W was significantly higher for both LO treatments (LO/LA & LO/MA), compared to growth

at the same ammonia level, but at a higher oxygen saturation (MO/LA & MO/MA). It is possible that oxygen levels in the LO treatments were sufficiently low for the abalone to fully activate the biochemical response to hypoxia. In this case, once phosphoarginine reserves in the pedal muscle were exhausted, glycogen and aspartate would become substrates for anaerobic glycolysis (Gäde 1983; Gäde & Ellington 1983; Hochachka *et al.* 1983; Gäde 1988). Movement of haemolymph into the pedal muscle would be limited (Jorgensen *et al.* 1984; Russell & Evans 1989), thereby conserving available oxygen for sensitive tissues such as heart, digestive gland and kidney (Russell & Evans 1989; Wells *et al.* 1998). Utilizing this strategy may sacrifice growth in the short term but would increase the chance of survival in the longer term.

Oxygen saturation levels below 80% reduces heart rate in molluscs (Bayne 1971; Nimura & Yamakawa 1989) including abalone (Nakanishi 1979; Russell & Evans 1989). Since the rate of gill perfusion is a function of heart rate, exposure to the reduced oxygen levels in the LO treatments would potentially reduce the amount of ammonia transferred across branchial tissue, in turn reducing the energy required to cope with environmental ammonia exposure. This may allow for improved growth rates compared to ammonia exposure at a higher level of DO, where little or no change in branchial perfusion would be expected.

Oxygen consumption data from Harris *et al.* (1999a) indicate that overall metabolic rate is reduced as hypoxia increases, which would further conserve the limited amount of oxygen available. Low DO has also been shown to reduce heart rate in several abalone species (Nakanishi 1978; Russell & Evans 1989), and this may have provided some protection for internal tissues in this trial in the LO treatments, by reducing gill perfusion and thus uptake across branchial membranes.

At MO oxygen levels, it is possible that the abalone are unable to fully develop these mechanisms, and the pedal muscle is still receiving a substantial portion of the available oxygen and attempting to function aerobically in an oxygen limited environment. This may incur a greater energetic cost than a fully hypoxic state. Oxygen consumption data for greenlip abalone suggest an increase in metabolic rate at

the ammonia levels in the MO/MA and MO/HA treatments (Harris *et al.* 1998a). There is evidence that ammonia exposure in freshwater mussels and prawns also increases the metabolic rate (Chen & Nan 1993; Chetty & Indira 1995). It is not clear whether this is a direct result of ammonia toxicity, or a secondary effect as part of the physiological response to elevated environmental ammonia levels.

As discussed in the Introduction, several authors have found that low oxygen levels combined with environmental ammonia increases the toxicity of the ammonia, at least in acute exposures. While this was also shown for elevated environmental ammonia levels in the presence of modest declines in oxygen saturation, the potential for very low DO levels to ameliorate the effect of environmental ammonia has not previously been reported.

The pattern of mortality between treatments following the hypoxic episode on Day 51 (Table 3.3) suggests that the highest ammonia exposure resulted in some degree of protection from this stress event as mortality was lower for both species than at lower TA. Presumably, the metabolic adaptations activated by the chronic exposure to ammonia combined with low DO allowed the abalone to better withstand extremely low DO. This further highlights the fact that abalone in this trial seem to have developed a metabolic strategy that sacrificed growth in the short term, but improved survival. The results from this event also demonstrate the remarkable tolerance of abalone to acute hypoxia, in that while there was substantial mortality among greenlip abalone there were still survivors in conditions bordering on anoxic. Blacklip abalone showed much lower mortality rates, indicating they are more tolerant of acute exposure to low dissolved oxygen levels than greenlip abalone. Given that the decline in growth rate found in the MO/HA treatment indicates that these abalone were already under some degree of stress, the ability to survive the hypoxic episode after the chronic exposure is even more remarkable.

Following the chronic exposure samples for serum ion analysis were taken from abalone in the control and three MO treatments. Chapter 4 presents the findings from these analyses, and shows that disruption in ion regulation may have contributed to the observed reduction in growth rates for both species.

### 3.5 Conclusions

The results show that growth was significantly depressed by chronic exposure to combinations of low dissolved oxygen and elevated ammonia levels with evidence that low levels of ammonia combined with modest declines in DO have a greater impact than either environmental ammonia or low DO alone. Growth at very low oxygen levels was higher than growth rates at the same environmental ammonia level combined with moderate oxygen levels, in particular in terms of weight. An hypoxic episode on Day 51 demonstrated that both species are able to withstand almost anoxic conditions, with blacklip abalone more resistant than greenlip abalone. This event also suggests that pre-exposure to high ammonia levels ameliorated the effects of exposure to low DO.

Overall, it is evident that the abalone in this trial responded to the chronic stress of the experimental treatments with a metabolic strategy that may have reduced growth rates, but increased the chance of long term survival.

Treatment	% Oxygen Saturation	DO (mg.L <sup>-1</sup> )	TA (mg.L <sup>-1</sup> )	UIA (µg.L <sup>-1</sup> )	pH	Nitrite (mg.L <sup>-1</sup> )
Control	96±0.5 <sup>a</sup>	7.16±0.03	0.24±0.02 <sup>a</sup>	5.23±0.40	7.90±0.01	0.00±0.00
MO/LA	83±2 <sup>b</sup>	6.19±0.12	1.06±0.05 <sup>b</sup>	24.04±1.17	7.91±0.00	0.93±0.08
MO/MA	76±1 <sup>c</sup>	5.58±0.07	1.88±0.09 <sup>c</sup>	40.02±2.33	7.84±0.01	1.33±0.09
MO/HA	85±2 <sup>b</sup>	6.27±0.16	8.07±0.20 <sup>d</sup>	197.25±9.77	7.92±0.01	1.10±0.13
LO/LA	64±2 <sup>d</sup>	4.89±0.10	1.44±0.04 <sup>b,c</sup>	38.70±0.81	7.98±0.00	0.40±0.06
LO/MA	56±1 <sup>d</sup>	4.29±0.09	2.48±1.08 <sup>e</sup>	59.01±2.50	7.91±0.00	1.08±0.03

Table 3.1 Measured water quality during growth trial for one control and 5 experimental treatments (excluding hypoxic event on Day 51, which is shown in Table 3.2) (mean±SE, n=3 for control & MO treatments, n=2 for LO treatments). DO, pH and temperature were measured daily; samples for analysis of TA were taken daily from 2 of the replicate tanks on each treatment as described in the text. Nitrite was samples were taken every 7-10 days. The data for each replicate tank was averaged for statistical analysis. Mean temperatures ranged from 18.54 – 19.12 °C for each treatment (n=3). Different superscripts indicate significantly different means (P<0.001).



Species	Parameter	n	F Ratio	DF	P
	Dissolved Oxygen (% Saturation)	2-3	78.012	5, 10	0.000
	Total Ammonia	2-3	738.009	5,10	0.000
Blacklip	SGR-L	21-43	5.722	5, 189	0.000
	SGR-W	21-40	15.1578	5, 175	0.000
Greenlip	SGR-L	25-39	20.224	5, 186	0.000
	SGR-W	14-39	30.926	5, 141	0.000
	L1:W1	14-39	5.074	5, 141	0.000

Table 3.2 Data from statistical analyses of water quality and growth data. The data were checked for homogeneity of variance by observation of residual plots generated using JMP 3.2. Using SPSS 10.02, ANOVA was used to determine the presence of significant treatment effects, with Tukeys HSD used to compare individual treatments. Analysis of water quality data was based on means for individual replicate tanks. The analysis of growth data used the individual tagged survivors in each treatment, with replicate nested within treatment. The water quality data are shown in Table 3.1, and growth data in Figures 3.1 & 3.2.

Treatment	Dissolved Oxygen		Ammonia		Greenlip		Blacklip	
	mg.L <sup>-1</sup>	% saturation	TA (mg.L <sup>-1</sup> )	UIA (µg.L <sup>-1</sup> )	Mortality Count*	% Mortality	Mortality Count*	% Mortality
Control	7.42±0.13	99±1	0.52±0.13	9.02±2.74	0,0,0	0	0,0,0	0
MO/LA	0.39±0.20	5±2	0.17±0.02	0.72±0.22	12,8,0	44±41	2,2,1	11±4
MO/MA	0.47±0.04	6±1	1.37±0.10	4.95±0.20	9,9,10	62±9	3,1,2	13±7
MO/HA	0.48±0.09	6±1	7.25±0.88	31.69±1.84	6,0,0	13±23	2,1,0	7±6

Table 3.3 Water quality and mortality data from an hypoxic event on Day 51 (means±SE, n=3), when a technical error prevented the bioassay units on these treatments from receiving influent water for 20 hours. Water was sampled at the time the error was discovered. The control units were aerated, experimental units were not. Temperature ranged from 17.26-17.96°C at the end of the hypoxic episode. pH ranged from 7.13-7.21 for experimental treatments, and was 7.81 for control. Treatment codes are explained in Table 3.1.

\* Figures from individual replicate tanks.

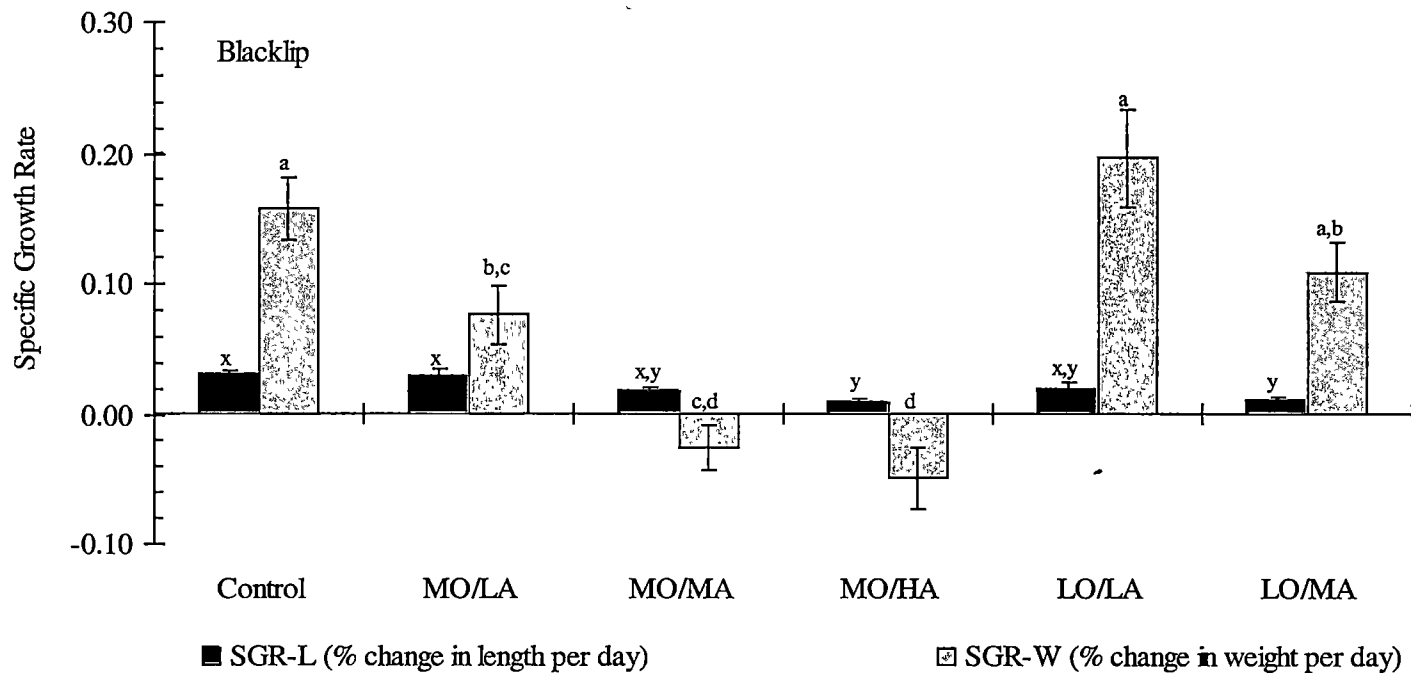


Figure 3.1 Growth data for blacklip abalone (means $\pm$ SE, n values given in Table 3.2) as specific growth rates (% change per day). Different superscripts indicate significantly different means ( $P < 0.001$ ). Water quality data for the experimental treatments are given in Table 3.1. Growth was monitored for up to 67 days as described in the main text. Statistical analysis was based on all the tagged survivors in each treatment rather than replicate means.

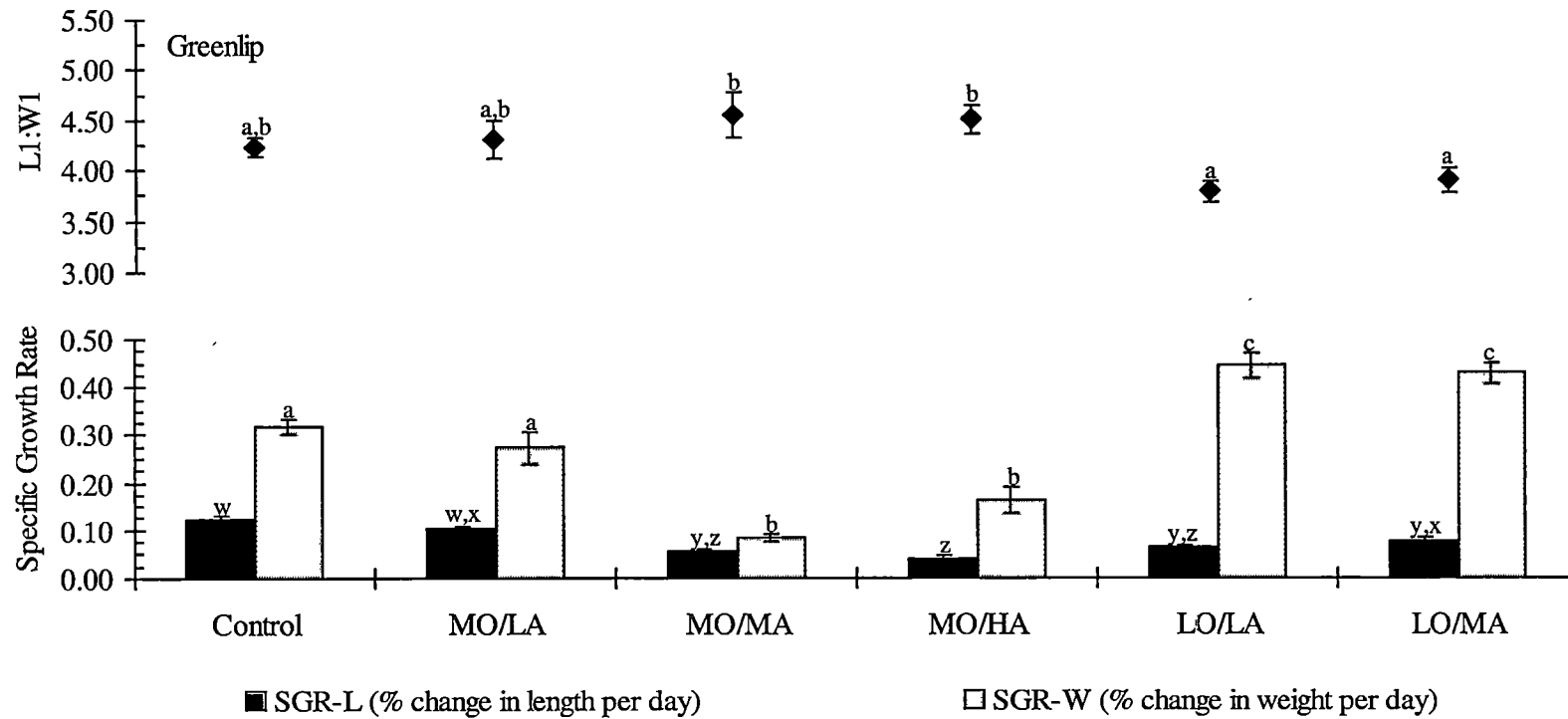


Figure 3.2 Growth data for greenlip abalone (means±SE, n values given in Table 3.2). Different superscripts indicate significantly different means ( $P<0.001$ ). Water quality data for the experimental treatments are given in Table 3.1. Growth was monitored for up to 67 days as described in the main text. Statistical analysis was based on all the tagged survivors in each treatment rather than replicate means. L1:W1 is the length:weight ratio at the end of the growth trial, as described in the text. There were no significant effects on length:weight ratio between treatment at the start of the growth trial. Statistical analysis was based on all the tagged survivors in each treatment.

## CHAPTER 4

### Effect Of Chronic And Acute Exposure To Environmental Ammonia On Haemolymph Ions

#### 4.1 Introduction

Ammonia is the natural by-product of many intracellular biochemical processes in virtually every known species, and is also required for some anabolic processes. Every living organism is therefore adapted to deal with a normal biological level of ammonia. Haemolymph ammonia levels have been reported as  $2.1 \text{ mg.L}^{-1}$  in the marine bivalve *Atrina pectinata* (Suzuki 1988), and in crustaceans ranges from  $1.82\text{-}13.23 \text{ mg.L}^{-1}$  (Chen *et al.* 1994). In marine teleosts, removal of excess ammonia under normal conditions is generally considered to be a primarily passive process by diffusion down a concentration gradient (Evans & More 1988; Evans *et al.* 1989), and this is likely to be true in marine molluscs as well. While the neutral  $\text{NH}_3$  molecule is probably the main form of ammonia lost by diffusion directly through branchial tissue, being more soluble in the lipid-rich cellular membranes, there is evidence that leaky paracellular junctions found in the branchial tissue of marine organisms may be more permeable to  $\text{NH}_4^+$  than the tight junctions typical of freshwater species (Evans & More 1988; Evans *et al.* 1989; Wilson & Taylor 1992) (see Figure 4.1). Active transport mechanisms are also known to exist in teleosts (Evans & Cameron 1986; Evans *et al.* 1989; Wilkie 1997), crustaceans (Hunter & Kirschner 1986; Towle & Holleland 1987; Chen & Nan 1992b) annelids (Mangum *et al.* 1978) and marine molluscs (Mangum *et al.* 1978) (Figure 4.1). While only small amounts of ammonia are known to be excreted through the kidney in marine teleosts (Evans & More 1988), Suzuki (1988) reported substantial amounts of ammonia in kidneys of *Atrina pectinata*.

It is well known that exposure to elevated environmental ammonia concentrations leads to an increase in serum ammonia levels for both teleosts (Wilson & Taylor 1992; Knoph & Thorud 1996) and invertebrates (Chen & Cheng 1993; Chen *et al.* 1994; Schmitt & Uglow 1997) (Figure 4.7). The excess internal ammonia can be actively excreted against the concentration gradient at an energetic cost and serum ammonia levels will stabilize at a point dependent on the capacity of the active excretion mechanism and severity of the exposure conditions (Schmitt & Uglow 1997). In a study of the European shore crab (*Carcinus maenas*), Spaargaren (1990) reported that removal of ammonia from the haemolymph by passive diffusion ceases above environmental ammonia concentrations of  $4.2 \text{ mg NH}_4^+ \cdot \text{L}^{-1}$ . At higher levels, the concentration gradient is reversed and ammonia will diffuse inwards from the environment.

Elevated plasma ammonia levels are also likely to result in elevated cytoplasmic levels of ammonia, through a combination of active transport and diffusion of  $\text{NH}_3$  (Figure 4.7). This leads to disturbances in glycolysis and energy metabolism for both teleosts and molluscs (Sousa & Meade 1977; Arillo *et al.* 1981; Dabrowska & Wlasow 1986; Begum 1987; Jeney *et al.* 1992; Chetty & Indira 1995; Vedel *et al.* 1998). Ammonia exposure has also been linked to an increase in serum pH with both metabolic and respiratory origins (Twitchen & Eddy 1994; Schmitt & Uglow 1997; El-Shafey 1998).

Environmental factors that affect energy intensive processes such as ion regulation will result in a depletion of ATP reserves. In the case of ammonia, this would be exacerbated by disruption of the processes which generate ATP (Arillo *et al.* 1981; Chetty & Indira 1995; Vedel *et al.* 1998). Although abalone can produce ATP anaerobically using glycogen and aspartate as substrates, the rate of production is lower than for aerobic metabolism (Gäde & Grieshaber 1986). Extended exposure to environmental ammonia may thus lead to secondary effects such as growth reduction or disturbances in ion regulation. Exposure to elevated environmental ammonia levels are known to reduce growth rates in several species of abalone (Harris *et al.* 1998a; Basuyaux & Matthieu 1999).

Very little work has been done on factors that influence haemolymph chemistry and ion exchange in invertebrates, and even less on molluscs. Based

on studies with selective membrane transport inhibitors such as ouabain and amiloride (Mangum *et al.* 1978; Hunter & Kirschner 1986), and isolated membrane vesicles (Hunter & Kirschner 1986), it is known that invertebrates including molluscs have some but not all of the active transport mechanisms present in vertebrates (Figure 4.1). It is known that molluscs have a strong buffering system to cope with pH changes during hypoxic episodes, and carbonates from tissue stores or the shell can be mobilized to supplement the buffering mechanism (Burton 1983).

As described in Chapter 3, a growth trial investigated the effects of low dissolved oxygen levels in conjunction with elevated environmental ammonia levels. The focus of this chapter are haemolymph samples collected following this growth trial to investigate the effects of chronic exposure to ammonia, combined with 80% oxygen saturation, on haemolymph chemistry. Five to 10 abalone from each of two replicates were transferred to a separate system at the conclusion of the growth trial, without being sampled for haemolymph, and acutely exposed to high ammonia levels in conjunction with severely reduced dissolved oxygen levels in order to investigate the effects of such an acute exposure on haemolymph chemistry, and the influence of exposure history on these effects.

## 4.2 Methods & Materials

This trial generated data from two groups of abalone. The chronic exposure groups were held at 3 different levels of environmental ammonia (1.06, 1.88 & 8.06 mg.L<sup>-1</sup> TA) for up to 67 days at 80% oxygen saturation (as described in Chapter 3), plus a control held at 100% oxygen saturation with no added ammonia. Following this chronic exposure, 5-10 abalone from each of two replicates were removed to a separate system and, following an acclimation period of 36-48 hours, exposed for 8 hours to a much higher level of ammonia (14-21 mg.L<sup>-1</sup> TA) combined with low dissolved oxygen (30-40% saturation). This subset of the chronically exposed population is the basis of the acute exposure group.

Growth and water quality data from the chronic exposure is presented in detail in Chapter 3, and summarized in Figure 4.2 (growth), and Table 4.1 (water quality).

#### ***4.2.1 Acute Exposure***

At the end of the chronic exposure, 5-10 abalone (none of which had previously been sampled for haemolymph) from each of two replicates were transferred to separate chambers in a respirometer. The respirometer system used 2.3 L Perspex chambers, which were supplied on a flow-through basis from one of the reservoirs used in the growth trial. Ammonia was added to the reservoir as for the chronic exposure trial to achieve the required TA concentration. The abalone were held at the experimental ammonia and oxygen levels for 36-48 hours to allow recovery from the transfer (Table 4.1). On Day 3, the conditions were altered abruptly as shown in Table 4.1 for 8 hours, starting between 8.00-9.00 am. Water flow through the chambers was stopped and the reservoir supplying the acute exposure system was drained, refilled and  $\text{NH}_4\text{Cl}$  added to achieve the required TA level for the acute condition. Water flow was restored within 10-15 minutes. The reduction in dissolved oxygen level was achieved by increasing the volume of nitrogen being passed into the mixing chamber. Following the 8 hour exposure, the abalone were returned to the experimental conditions for a further 36-48 hours. After this time, the haemolymph was sampled as described below. A concurrent trial using oxygen consumption to investigate abalone metabolism in during and after a stress event meant that the abalone could not be disturbed immediately following the acute exposure. The abalone were in the respirometer for a total of 4 days.

Water samples were taken daily, and once during the acute exposure, for analysis of ammonia as described in Chapter 3. DO, pH and temperature were recorded daily as described in Chapter 3. During the acute exposure, pH was recorded once, and DO was monitored every 30-40 minutes.

#### ***4.2.2 Haemolymph Sampling***

At the end of the growth trial, haemolymph was sampled from the cephalic arterial sinus, centrifuged at 13 000 rpm for 5 minutes as per Harris (1999) and



frozen in a fresh Eppendorf tube for subsequent analysis. Haemolymph pH was determined prior to centrifugation, in the Eppendorf tube using a TPS probe with a 4 mm tip.

Haemolymph cations were analysed after the appropriate dilution with de-ionised water by atomic absorption spectroscopy. Haemolymph chloride was determined using the method of Zall *et al.* (1956).

#### ***4.2.3 Effect of Starvation on Haemolymph Ions***

As the abalone used in the acute challenge were not fed after being transferred from the system used for the chronic exposure, a trial was conducted to determine any effects of starvation on serum ions. Blacklip and greenlip abalone from the same group used in the trial were sampled after being fed normally, or being starved for 4 days. Separate groups were used for each sample, and each abalone was only sampled once. The haemolymph samples were treated and analysed as for the other samples. This trial was conducted after the chronic exposure trial, and the abalone had been held at ambient conditions for 3-4 weeks (90-100% oxygen saturation, no detectable ammonia, pH 8.1-8.2, 16-18°C).

Where significant effects of starvation were observed (based on ANOVA), a correction factor was derived by dividing the mean result for fed abalone by the mean result for starved abalone. The ion data from the acute exposure were then multiplied by this correction factor prior to statistical analysis. This transformation presented the data on the basis that the abalone had been fed during the acute exposure.

#### ***4.2.4 Statistical Analysis***

All data were checked for homogeneity of variance by observation of residual plots generated by JMP 3.2. No transformations were required. In analysing the haemolymph ion data, results from all the individual abalone on each treatment were used rather than the means of individual replicates. SPSS (SPSS Inc.) was used to analyse the data, with any treatment effects determined

using ANOVA (with replicate nested within treatment to allow for any variation between replicates), and comparing individual treatments with Tukeys HSD. The F ratio, degrees of freedom and P value are given for significant differences in Table 4.2 (chronic exposure) and Table 4.3 (acute exposure). SPSS was used for these analyses. Students t-tests were used to determine the effect of the challenge compared to the chronic exposure for individual treatments, using JMP 3.2, and the results are shown in Table 4.4.

The significance of any effect from starvation on haemolymph ions was determined by ANOVA and comparing individual means with Tukeys HSD using JMP 3.2.

### 4.3 Results

Water quality data from the chronic and acute exposure trials are given in Table 4.1. Growth data generated by the chronic exposure is summarized in Figure 4.2, and fully presented in Chapter 3. The effects on the haemolymph ions following the chronic exposure are summarized in Table 4.2 with the statistical data, and shown in Figures 4.4, 4.5 and 4.6. Results from the acute exposure are shown Table 4.3. The comparison between chronic and acute exposure is given in Table 4.4. However, although the treatment effects followed a similar pattern in both species, the effects tended to be significant in blacklip rather than greenlip abalone.

#### *4.3.1 Effects of Starvation*

There was a significant effect of starvation on haemolymph levels of sodium for blacklip abalone (32.198, 1, 18,  $P < 0.001$ ) (by a factor of 1.2), and potassium and calcium in greenlip abalone (factors of 0.8 and 1.1 respectively) (potassium – 25.155, 1, 18,  $P < 0.001$ ; calcium – 14.681, 1, 18,  $P < 0.001$ ) (Figure 4.3). Since the abalone from the chronic trial were fed, while no food was provided during the acute exposure trial, the raw data were adjusted for these effects prior to statistical analysis, and the discussion below is based on the adjusted data.

### 4.3.2 Haemolymph pH and Calcium

Haemolymph pH in greenlip abalone from the MO/HA treatment was significantly higher than from the other treatments (17.927, 3, 44,  $P<0.001$ ), which did not differ (Figure 4.4A, Table 4.2). In blacklip abalone, haemolymph pH was significantly lower in the MO/MA treatment than from abalone in the other treatments (29.111, 3, 44,  $P<0.001$ ), which did not differ significantly.

For both species and all treatments, haemolymph pH was significantly higher following the acute exposure compared to the chronic exposure (blacklip –  $t=3.65$ , 13,  $P=0.005$ ; greenlip –  $t=5.541$ , 29,  $P<0.001$ ) (Figure 4.4A, Table 4.4). After the acute exposure in greenlip abalone, haemolymph pH was significantly higher in the MO/LA, MO/MA and MO/HA treatments than for the control (6.436, 3, 26,  $P=0.002$ ).

Following the chronic exposure in blacklip abalone, haemolymph calcium levels were significantly higher in the MO/LA and MO/MA treatments than in the MO/HA treatment (16.627, 3, 43,  $P<0.001$ ) (Figure 4.4B, Table 4.2). Haemolymph calcium in abalone from the MO/MA treatment were also significantly higher than in control abalone.

Although haemolymph calcium levels in greenlip abalone were highest from the MO/MA treatment as in blacklip abalone, there were no significant treatment effects in greenlip abalone ( $P>0.05$ ) (Figure 4.4B).

After the acute exposure, haemolymph calcium levels in both species were significantly higher in MO/LA and MO/MA treatments than the control and MO/HA treatments (blacklip – 31.751, 3, 31,  $P<0.001$ ; greenlip – 7.525, 3, 22,  $P=0.001$ ) (Figure 4.4B, Table 4.3). Compared to haemolymph calcium levels from the chronic exposure, haemolymph levels were significantly higher in the MO/LA, MO/MA and MO/HA treatment for greenlip abalone ( $t=2.252$ , 14,  $P=0.024$ ) (Table 4.4).

### 4.3.3 Haemolymph Magnesium, Potassium & Sodium

Figure 4.5 shows the results for haemolymph magnesium, potassium and sodium. In chronically exposed blacklip abalone, magnesium levels in the MO/LA treatment were significantly higher than for control and MO/HA treatments (11.326, 3, 43,  $P<0.001$ ) (Figure 4.5A, Table 4.2), with intermediate

levels observed from the MO/MA treatment which were similar to the control. A similar pattern was observed for haemolymph potassium levels in blacklip abalone, with levels from the MO/LA treatment being significantly higher than in abalone from the MO/MA and MO/HA treatments (9.704, 3, 43,  $P<0.001$ ) (Figure 4.5B, Table 4.2). Haemolymph potassium levels in control blacklip abalone were not different from abalone in the MO/LA treatment, but were significantly higher than from the MO/HA treatment. Haemolymph sodium levels from blacklip abalone in the MO/MA and MO/HA treatment were significantly lower than in control abalone (4.468, 3, 41,  $P=0.008$ ) (Figure 4.5C, Table 4.2), but the MO/LA treatment did not differ from the control.

There were no significant effects on haemolymph magnesium, potassium or sodium levels in greenlip abalone following the chronic exposure, either between the experimental treatments or compared to the control (Figure 4.5, Table 4.2).

Following the acute exposure in blacklip abalone, haemolymph magnesium levels were significantly higher in abalone from the control and MO/LA treatments compared to abalone from the MO/HA treatment (11.851, 3, 31,  $P<0.001$ ) (Figure 4.5B, Table 4.3). Haemolymph magnesium levels from the control treatment were also significantly higher than from the MO/MA treatment. In blacklip abalone following the acute exposure haemolymph levels of both potassium and sodium were significantly higher in control abalone than the three experimental treatments (potassium – 6.803, 3, 31,  $P=0.001$ ; sodium – 13.929, 3, 31,  $P<0.001$ ), which did not differ (Figure 4.5B,C, Table 4.3).

Compared to haemolymph levels from chronic exposure in blacklip abalone, haemolymph magnesium and potassium levels were significantly higher in the control treatment following the acute exposure (magnesium –  $t=4.798$ , 11,  $P=0.001$ ; potassium – 3.649, 11,  $P=0.004$ ) (Figure 4.5A,B; Table 4.4). In terms of sodium, haemolymph levels after the acute exposure were significantly higher in blacklip abalone from the control ( $t=10.660$ , 11,  $P<0.001$ ), MO/MA ( $t=16.728$ , 23,  $P<0.001$ ) and MO/HA treatments compared to the chronic exposure ( $t=12.460$ , 19,  $P<0.001$ ) (Figure 4.5C, 4.4).

Haemolymph sodium and magnesium levels in greenlip abalone after the acute exposure were not significantly different from levels following the chronic

exposure ( $P>0.05$ ). Haemolymph potassium levels from control abalone were significantly higher than in abalone from the MO/HA treatment (3.968, 3, 22,  $P=0.021$ ), which did not differ significantly from the other experimental treatments (Figure 4.5B, Table 4.3). Following the acute exposure in greenlip abalone, haemolymph potassium levels were significantly lower in the control and experimental treatments than after the chronic exposure (Figure 4.5C, Table 4.4).

#### ***4.3.4 Haemolymph Copper***

For blacklip abalone, haemolymph copper levels in control abalone following the chronic exposure were significantly higher than in abalone from the MO/HA treatment (2.503, 3, 43,  $P=0.072$ ), but did not differ significantly from the other treatments (Figure 4.6A, Table 4.2). There were no significant differences ( $P>0.05$ ) between treatments and/or controls in blacklip abalone following the acute exposure to environmental ammonia ( $P>0.05$ ).

Haemolymph copper levels in greenlip abalone from the MO/MA treatment were significantly lower than in control abalone (4.278, 3, 40,  $P=0.010$ ), but did not differ significantly from abalone in the other experimental treatments (Figure 4.6A, Table 4.2).

#### ***4.3.5 Haemolymph Chloride***

In greenlip abalone after the chronic exposure, haemolymph chloride levels from control abalone were significantly lower than abalone from the MO/LA treatment (4.362, 3, 37,  $P=0.010$ ), but control abalone did not differ significantly from the other experimental treatments (Figure 4.6B, Table 4.2). There were no significant effects on haemolymph chloride levels in greenlip abalone following the acute exposure (Figure 4.6B, Table 4.3). Compared to haemolymph chloride levels following the chronic exposure, haemolymph levels after the acute exposure in control greenlip abalone were significantly higher ( $t=2.182$ , 23,  $P=0.039$ ) (Figure 4.6B, Table 4.4).

#### 4.4 Discussion

A large amount of work has been done on the effects of ammonia on haemolymph chemistry and ion regulation in freshwater species, but the impact of ammonia on marine organisms have been relatively poorly studied. Fundamental chemical differences between seawater and freshwater mean that exposure to environmental ammonia has substantially different effects in the two environments (Wilson & Taylor 1992; Lucu & Pavivic 1995). Unless otherwise specified the following discussion is limited to the marine environment.

The lipid solubility of neutral molecules such as  $\text{NH}_3$  and the predominance of un-ionized ammonia in most aquatic environments has traditionally implicated this form of ammonia as the primary toxic agent (Bower & Bidwell 1978; Wilkie 1987). It is increasingly evident that branchial tissue from marine organisms tends to be much more permeable to cations such as  $\text{NH}_4^+$  than previously considered, due to leaky paracellular junctions (Evans & Cameron 1989; Wilson & Taylor 1992; Wilkie 1997). It was therefore considered more appropriate to present and discuss the results in terms of TA rather than UIA.

Since the chronic exposure covered a period of up to 67 days, it is likely that chronically exposed abalone had achieved equilibrium with the altered environment and either recovered from any effects of, or acclimated to, the elevated environmental ammonia levels. The effects of acute exposure were determined from samples taken 36–48 hours following the exposure, with the abalone returned to the chronic exposure condition for the interim period. The delay in sampling was a constraint of a concurrent trial investigating the effect of ammonia exposure on metabolic rate which precluded sampling directly after the exposure. Where the results were similar to those following the chronic exposure, there was either no effect of the acute exposure or the abalone had recovered to the pre-existing condition. In cases where there was a difference between chronically and acutely exposed abalone the damage was either irreparable or recovery was still in progress.

Comparing the results for control and experimental treatments shows that chronic exposure to elevated environmental ammonia levels did have substantial effects on the haemolymph chemistry of both species. It is known that

invertebrates including molluscs have some but not all of the active transport mechanisms present in vertebrates (Mangum *et al.* 1978; Hunter & Kirschner 1986). Molluscs are also known to have a well developed buffering system to cope with pH changes during hypoxic episodes, and are capable of mobilizing carbonates from tissue stores or the shell matrix to supplement the buffering mechanism (Burton 1983). What is known from the literature and what is suggested by results from the current study is shown in Figure 4.7, which forms the basis of the following discussion.

There are very few data available on haemolymph ammonia levels in marine gastropods, but haemolymph ammonia levels in abalone are likely to be of a similar order to the marine bivalve *Atrina pectinata*, quoted by Suzuki (1988) as 2.1 mg TA.L<sup>-1</sup>. This is similar to the ammonia level from the MO/MA treatment (1.88 mg TA.L<sup>-1</sup>), and would be exceeded in the MO/HA treatment (8.06 mg TA.L<sup>-1</sup>) and the acute exposure (14-21 mg TA.L<sup>-1</sup>). Based on evidence from both vertebrate and invertebrate species (Wilson & Taylor 1992; Chen & Cheng 1993; Chen *et al.* 1994; Knoph & Thorud 1996; Schmitt & Uglow 1997), haemolymph ammonia levels in abalone from these treatments would therefore be expected to increase during the initial exposure to these environmental ammonia levels, primarily due to an increased influx from passive diffusion and inhibition of efflux by diffusion. In the MO/MA treatment, even though the passive process may not have ceased completely, the change in concentration gradient would mean that the rate of diffusion would have decreased and haemolymph ammonia would still accumulate. It would be expected that active excretion of ammonia would therefore increase to compensate for the decrease in diffusion, and haemolymph ammonia would eventually stabilise at a level higher than the pre-exposure level, but lower than from the initial exposure. It should be noted that while environmental ammonia levels in the MO/LA treatment would not be expected to directly increase haemolymph ammonia levels, there were still effects from this treatment following both the chronic and acute exposures in both species. This may be due to abalone naturally having very low levels of haemolymph ammonia, which would allow the low level of environmental ammonia in this treatment to alter the concentration gradient and thus affect the haemolymph ammonia level.

Harris *et al.* (1998b) reported damage to gill and kidney tissue in greenlip abalone following chronic exposure to elevated environmental ammonia and hypoxia as individual variables. As both organs are likely to be involved in active ammonia excretion (Suzuki 1988; Harris *et al.* 1998b), physical damage may impede this function. Ammonia is also known to compete with cations such as potassium for binding sites in active transport mechanisms (Towle & Holleland 1987), and thus elevated environmental ammonia levels would tend to displace the correct cation and increase the net influx of ammonia while also disrupting regulation of the cation involved.

Harris (1999) did not report any significant effect on haemolymph ions in greenlip abalone chronically exposed to hypoxia (down to 55% saturation). It is likely that healthy abalone would be able to cope with the hypoxic conditions of the acute exposure, given that abalone are physiologically suited to dealing with functional hypoxia (Gäde 1988). Chapter 2 also showed that blacklip and greenlip abalone otherwise held in normal seawater ( $<0.1 \text{ mg TA.L}^{-1}$ , 95-100 % DO saturation) showed no reduction in growth from intermittent exposure to 5  $\text{mg TA.L}^{-1}$  and 60 % DO saturation. Branchial damage has been reported after chronic exposure to 55% oxygen saturation in greenlip abalone and acute exposure to  $30.24 \text{ mg.TA l}^{-1}$  (Harris *et al.* 1998b), so it is possible that a degree of branchial damage could have resulted from the short term environmental alterations established for the acute exposures. Based on the study of Harris *et al.* (1998b), chronic exposure to 80% oxygen saturation would not be expected to result in branchial damage.

#### ***4.4.1 Haemolymph pH and Calcium***

Given that haemolymph pH in chronically exposed greenlip abalone from the MO/LA & MO/MA treatments did not differ from the control, it is considered that the alkalosis seen in the MO/HA treatment was due to the environmental ammonia exposure rather than the modest hypoxia. An increase in serum pH has also been reported in rainbow trout acclimated to seawater and exposed to ammonia for several days (Wilson & Taylor 1992), which was attributed to an increase in the base load due to  $\text{NH}_4^+/\text{Na}^+$  exchange. If a similar system was operating in abalone, it would increase the base load and hence



haemolymph pH in a similar manner (by exporting the base from the haemolymph in exchange for cations). While the expected increase in cations (magnesium, potassium or sodium) was not observed following the chronic exposure, other cations may be involved or ammonia is being exported by some other mechanism. The decrease in cations observed in chronically exposed blacklip abalone from the MO/HA treatment suggests a symporter may also be operating.

It has been suggested that the excess protons from the deposition of calcium carbonate in the molluscan shell or tissue carbonate stores may be removed by formation of  $\text{NH}_4^+$  from  $\text{NH}_3$  (Burton 1983; Wilbur & Saleuddin 1983). The normal equilibrium position would tend towards the formation of  $\text{NH}_4^+$ , but elevated haemolymph ammonia levels may alter this equilibrium, resulting in a build up of haemolymph carbonates and calcium. The formation of  $\text{NH}_4^+$  from  $\text{NH}_3$  in the haemolymph will also consume hydrogen ions and thus contribute to any increase in haemolymph pH as well (Twitchen & Eddy 1994; Schmitt & Uglow 1997; El-Shafey 1998; Schmitt & Santos 1999). Even though a larger percentage of ammonia diffuses into marine organisms as  $\text{NH}_4^+$  compared to freshwater, the predominant form will still be entering as  $\text{NH}_3$  (Evans & Cameron 1986). The un-ionised form will then be converted to the ionised form in the haemolymph, and actively exported.

Interference in shell formation by elevated haemolymph ammonia levels would also explain the increase in haemolymph calcium levels observed in the MO/LA and MO/MA treatments for both species (significant for both species). The low calcium level in the MO/HA treatment may indicate a more severe disturbance in this treatment (observed in both species, significantly lower than other experimental treatments for chronically exposed blacklip abalone). The growth data also shows that these abalone were more severely affected in terms of growth than those in the MO/LA and MO/MA treatments (Figure 4.2).

For both species and all treatments, there was a substantial increase in haemolymph pH following the acute exposure. This more severe and prolonged alkalosis may be an over-compensation for an acidosis due to the hypoxic episode or respiratory insufficiency due to branchial damage from the acute exposure condition. Both the environmental ammonia level and oxygen

saturation in the acute exposure have been found to cause damage to gill tissue in longer term exposures (Harris *et al.*, 1998b). It is also known that there may be quite an extensive recovery period following hypoxic exposures in molluscs, during which anaerobic metabolism may continue while ATP and phosphoarginine reserves are restored (Ellington 1983; Gäde & Grieshaber 1986). Such a recovery period may have contributed to the extended alkalosis observed in this trial.

Following environmental hypoxia the European shore crab was also shown to develop an alkalosis, which was controlled by several mechanisms including haemolymph  $\text{CO}_2$  levels and an change in the equilibrium between  $\text{HCO}_3^-$  and  $\text{CO}_3^{2-}$  (Johnson & Uglow 1987). These authors also reported an increase in haemolymph calcium levels in conjunction with the alkalosis, possibly due to dissolution of the exoskeleton to supply the carbonate buffer system. Molluscs are known to mobilize calcium and carbonates from the shell or tissue stores to buffer the acidic by-products of anaerobic metabolism (Burton 1983).

For molluscan haemocyanin an alkaline pH increases the affinity for oxygen as the oxygen tension decreases due to a reversal of the Bohr and Root shifts found in vertebrate species (Brix *et al.* 1979; Wells *et al.* 1998a) which would assist in maintaining oxygen reserves in the haemolymph for sensitive tissues such as heart, kidney and neural ganglia (Russell & Evans 1989; Wells *et al.* 1998a). It is possible that the alkalosis, at least in part, is deliberately sustained in order to maximize oxygen delivery to the tissues during the recovery period.

#### ***4.4.2 Haemolymph Magnesium, Potassium & Sodium***

In blacklip abalone after the chronic exposure, haemolymph levels of magnesium, potassium and sodium from the MO/HA treatment were all significantly lower than in control abalone. This may be due to a disturbance in cation regulation as a result of general branchial damage, or ammonia poisoning specific ion exchange mechanisms. It is also possible that ammonia is being co-transported outwards with one or more of these cations in a symporter rather than being exchanged, which is known for potassium and sodium in teleosts (Evans & Cameron 1986). Molluscs are known to have active sodium/ammonia

exchangers at least in the basolateral membranes (Figure 4.7), which operate at an energetic cost. Given that the treatments with the lowest haemolymph cation levels (Figure 4.5) also showed the lowest growth (Figure 4.2), it is possible that the overall cost of the ammonia exposure prevented the maintenance of normal ion exchange processes. It is also possible that a disturbance in regulation of calcium and magnesium may have interfered with shell formation, given the role these elements have in shell structure (Wilbur & Saleuddin 1983; McBean 1998).

Although the cation levels in greenlip abalone following the chronic exposure tended to follow the same general pattern as in blacklip abalone from the chronic exposure, there were no significant effects. This may be due to inter-specific differences, or the fact that the greater overall impact of the exposure conditions on blacklip abalone (evidenced by the lower growth rates) may have exaggerated the effect on haemolymph ions in this species compared to the greenlip abalone. The greenlip abalone may have been better able to maintain ion regulation, being in better physiological condition (which is suggested by higher growth rates than the blacklip abalone).

Significant increases in all three haemolymph cations were observed in control blacklip abalone following the acute exposure, compared to the chronic exposure. Haemolymph sodium levels were also higher in blacklip abalone from the MO/MA and MO/HA treatments after the acute exposure compared to the chronic exposure. The fact that haemolymph magnesium and potassium levels from experimental treatments were similar following both the acute and chronic exposures suggests that pre-exposure to ammonia may have ameliorated the impact of the acute exposure in terms of these two ions. Pre-exposure to sublethal ammonia levels has also been shown to mitigate the response in terms of oxygen consumption to a more severe exposure in *Penaeus chinensis* (Chen & Nan 1993), and osmoregulation in rainbow trout (Lloyd 1961). These findings, in conjunction with the literature, indicate that pre-exposure to environmental ammonia provides some form of protection from subsequent exposures, either by allowing for a quicker recovery or ameliorating the effects of the exposure through physiological adaptation.

For greenlip abalone, the substantial increase in haemolymph cations following the acute exposure in blacklip abalone was not observed.

Haemolymph magnesium and sodium levels followed a similar pattern to the chronic exposures, but haemolymph potassium levels were depleted following the acute exposure. The lack of impact on haemolymph magnesium and sodium levels may be a reflection of a greater tolerance to environmental ammonia in greenlip abalone, or a quicker recovery. The low haemolymph potassium levels compared to the chronic exposure suggests potassium is being co-transported out of the basolateral or apical membranes with ammonia, as documented in marine fish (Figure 4.7) (Evans & Cameron 1986; Evans *et al.* 1989; Wilkie 1997).

Knoph & Thorud (1996) reported serum levels of chloride and sodium were elevated for more than 3 days in Atlantic salmon acutely exposed to environmental ammonia. This response was coupled to an increase in serum pH which was a consequence of elevated plasma ammonia levels, and the resulting increase in active export of ammonia in exchange for sodium and chloride. This co-transport mechanism has been demonstrated in other teleosts (Evans *et al.* 1989; Wilson & Taylor 1992) in both basolateral (serosal) and apical (mucosal) membranes, where it is the primary route of active ammonia excretion (Evans *et al.* 1989; Wilkie 1997). Basolateral sodium/ammonia exchange mechanisms sensitive to ouabain have been found in crustaceans, annelids and molluscs (Mangum *et al.* 1978; Hunter & Kirschner 1986; Wilkie 1997). Hunter & Kirschner (1986) did not find such an exchange mechanism in the apical membranes of molluscs and annelids, which may indicate a different exchange mechanism is operating or that diffusion is more important in apical membranes.

It is well known that osmoconforming molluscs such as abalone tend to use organic molecules such as amino acids for osmoregulation rather than inorganic ions (Burton 1983; Somero & Bowlus 1983). However, potassium and sodium are known to be regulated in marine molluscs (Burton 1983) including abalone (Boarder 1997). Results from the chronic exposure show that there was some degree of disturbance in haemolymph ions from elevated environmental ammonia, compared to the controls with no added ammonia. The energy requirements in terms of ATP for these active ion exchange mechanisms is comparatively high (Spaargaren 1990; Lucu & Pavicic 1995), and ammonia is known to interfere with the glycolytic pathways that generate ATP (Arillo *et al.* 1981; Chetty & Indira 1995; Vedel *et al.* 1998). These factors combined may

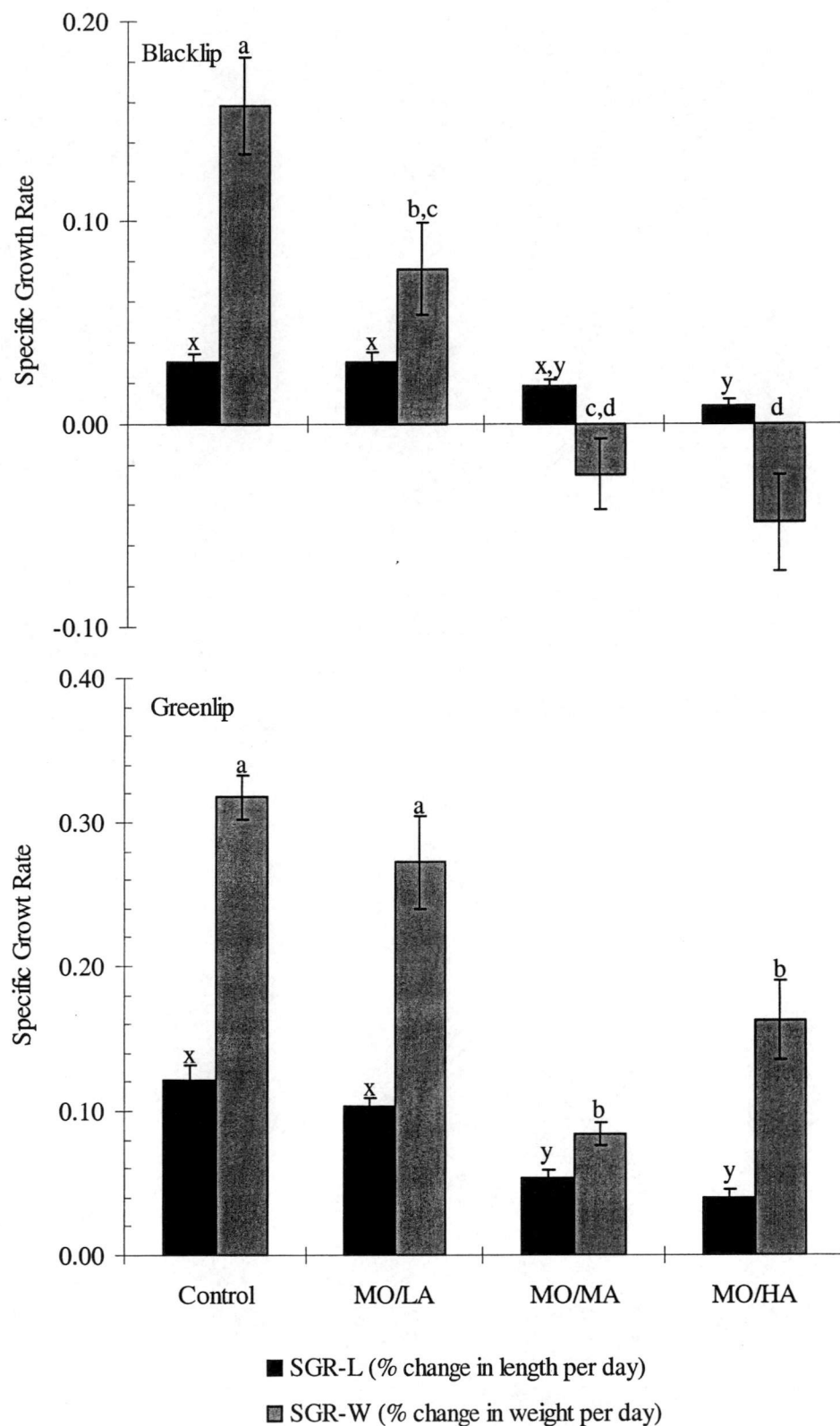


Figure 4.2 Growth data from chronic growth trial (modified from Chapter 3). Different superscripts indicate significantly different means ( $P < 0.001$ ). The chronic exposure ran for up to 67 days.

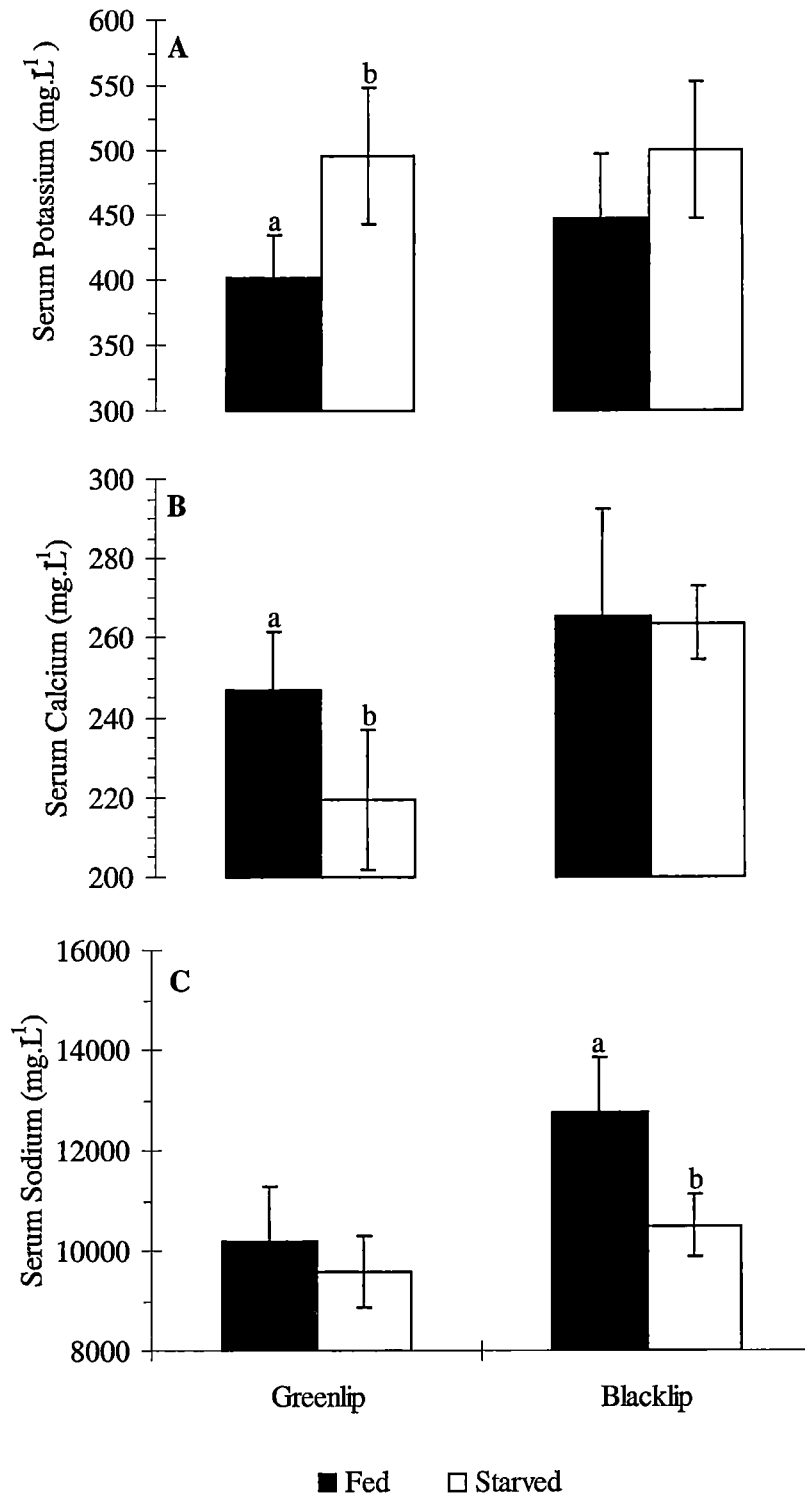


Figure 4.3 Effect of starvation on haemolymph sodium, calcium and potassium in greenlip and blacklip abalone. Different superscripts indicate significantly different means (means $\pm$ SD, n=40). The correction factors applied to the raw data for these ions were 1.2 for potassium in greenlip abalone, 1.1 for calcium in greenlip abalone and 0.8 for sodium in blacklip abalone

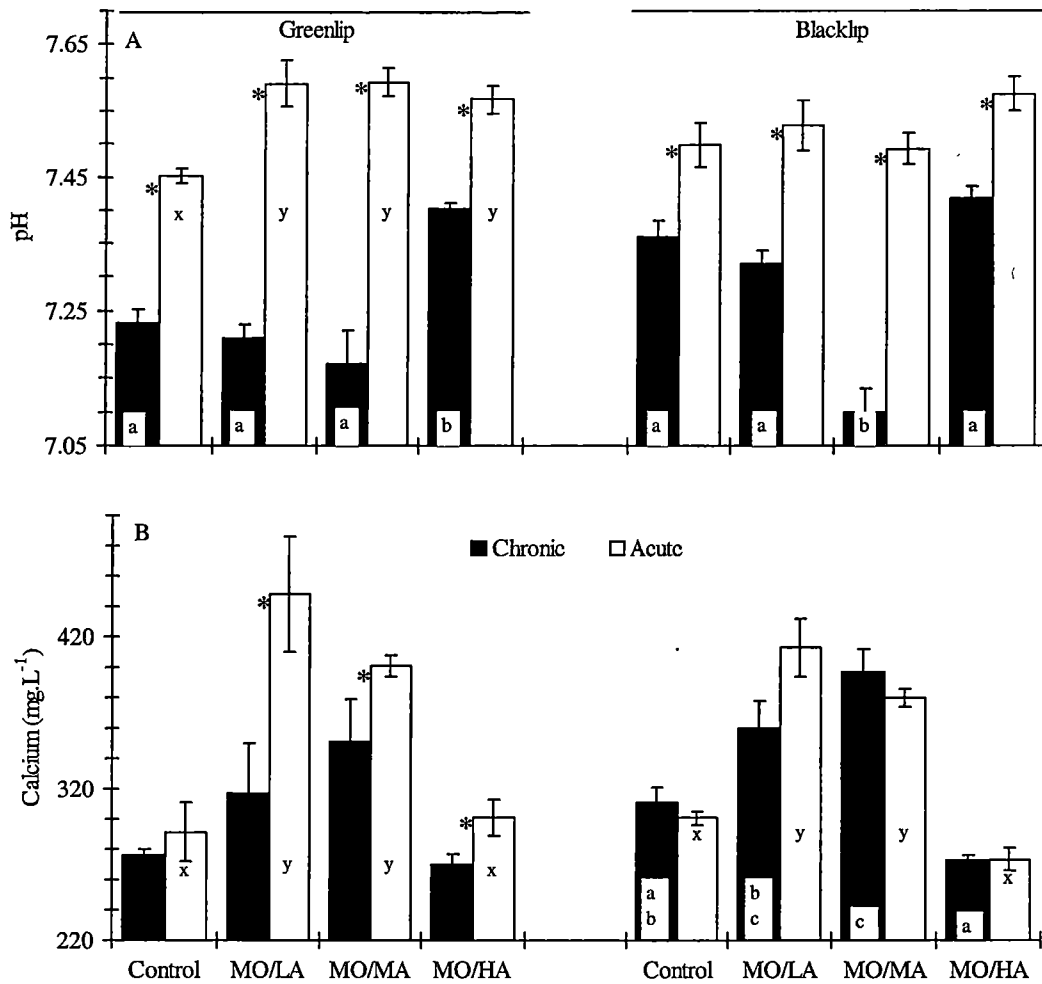


Figure 4.4 Haemolymph pH and calcium levels from chronic exposure to elevated environmental ammonia and acute exposure to environmental ammonia combined with hypoxia in greenlip and blacklip abalone (means $\pm$ SE, n values given in Table 4.2 (chronic) and 4.3 (acute)). Different superscripts indicate significantly different means ( $P < 0.05$ ). Asterix (\*) indicates a significant difference between chronic and acute exposures ( $P < 0.05$ ).

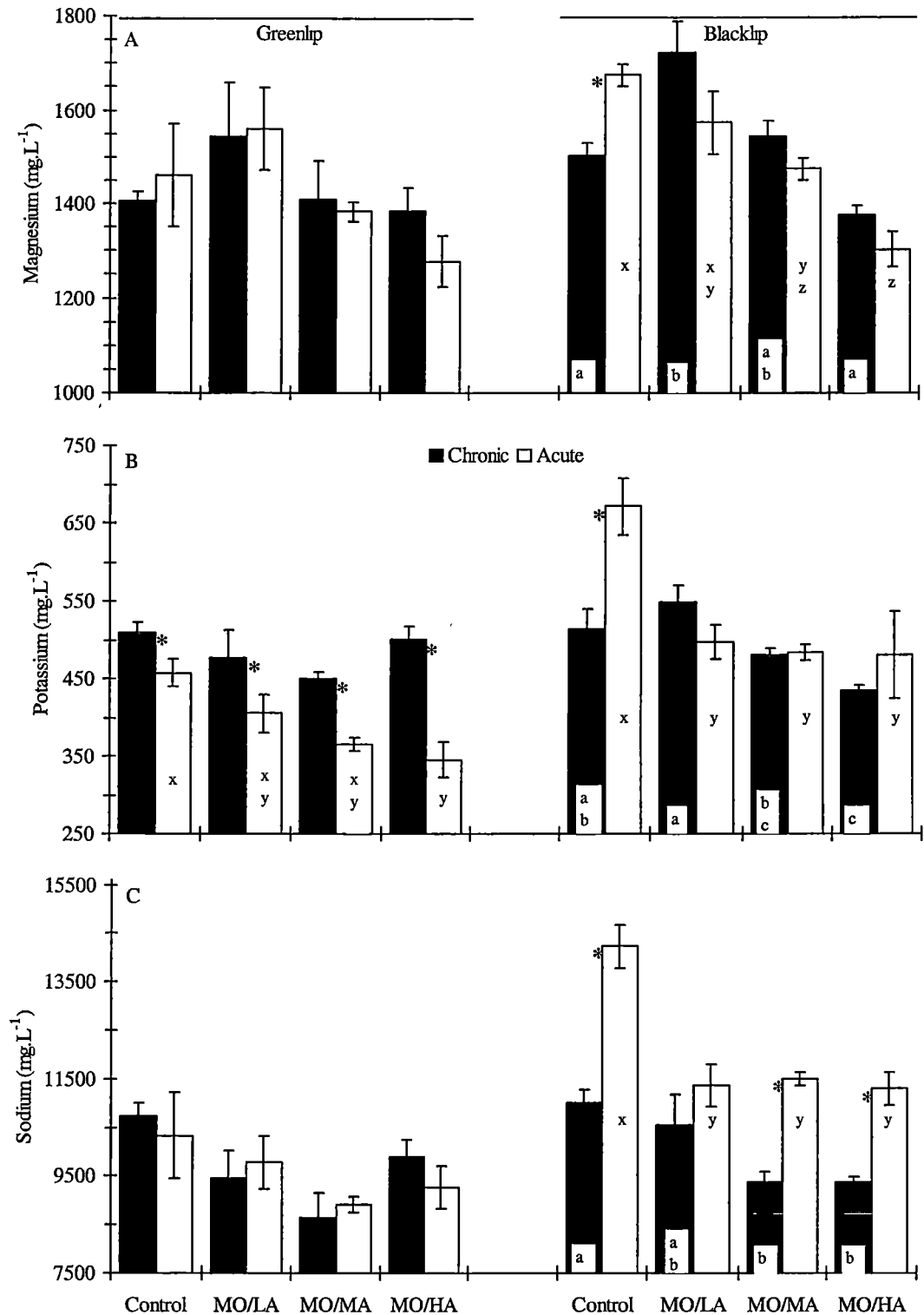


Figure 4.5 Haemolymph magnesium, potassium and sodium levels from chronic exposure to elevated environmental ammonia and acute exposure to environmental ammonia combined with hypoxia in greenlip and blacklip abalone (means $\pm$ SE, n values given in Table 4.2 (chronic) and 4.3 (acute)). Different superscripts indicate significantly different means ( $P < 0.05$ ). Asterix (\*) indicates a significant difference between chronic and acute exposures ( $P < 0.05$ ).



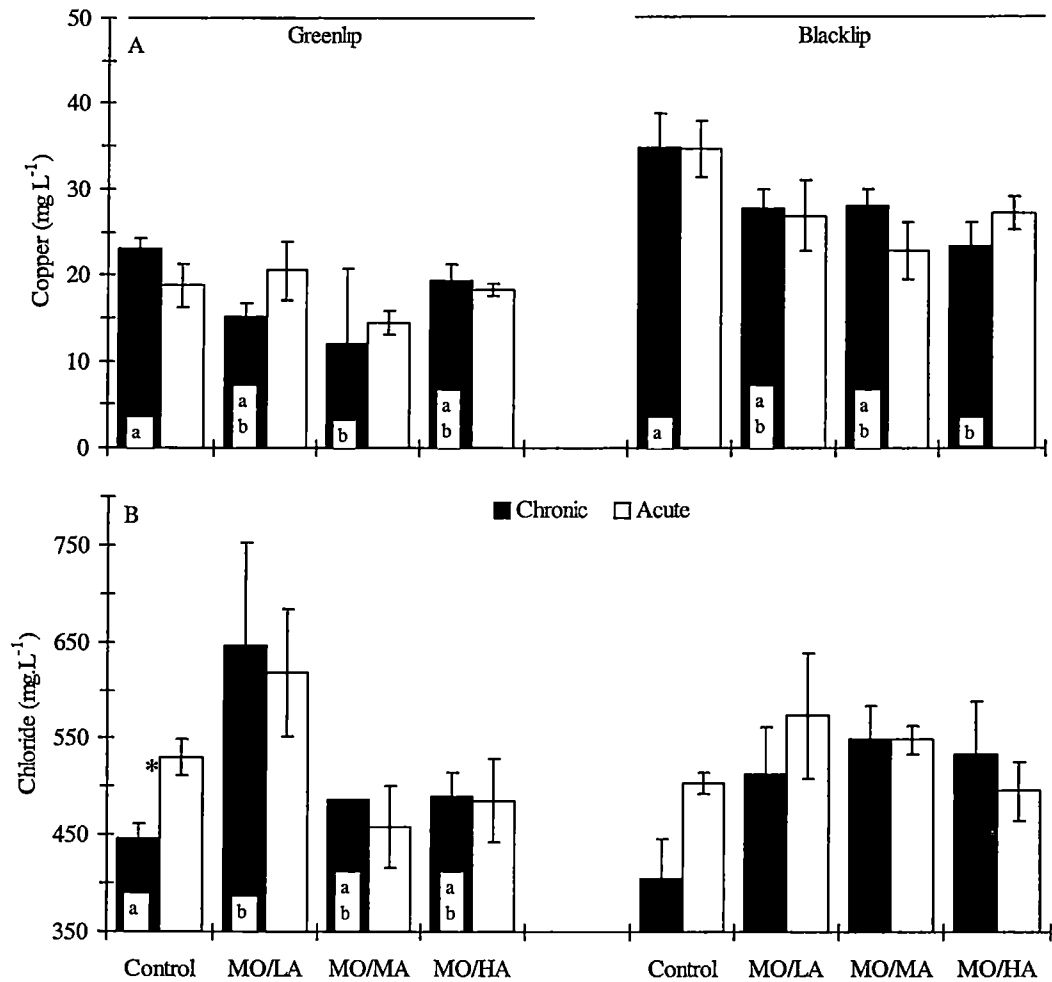


Figure 4.6 Haemolymph copper and chloride levels from chronic exposure to elevated environmental ammonia and acute exposure to environmental ammonia combined with hypoxia in greenlip and blacklip abalone (means $\pm$ SE, n values given in Table 4.2 (chronic) and 4.3 (acute)). Different superscripts indicate significantly different means ( $P < 0.05$ ). Asterix (\*) indicates a significant difference between chronic and acute exposures ( $P < 0.05$ ).

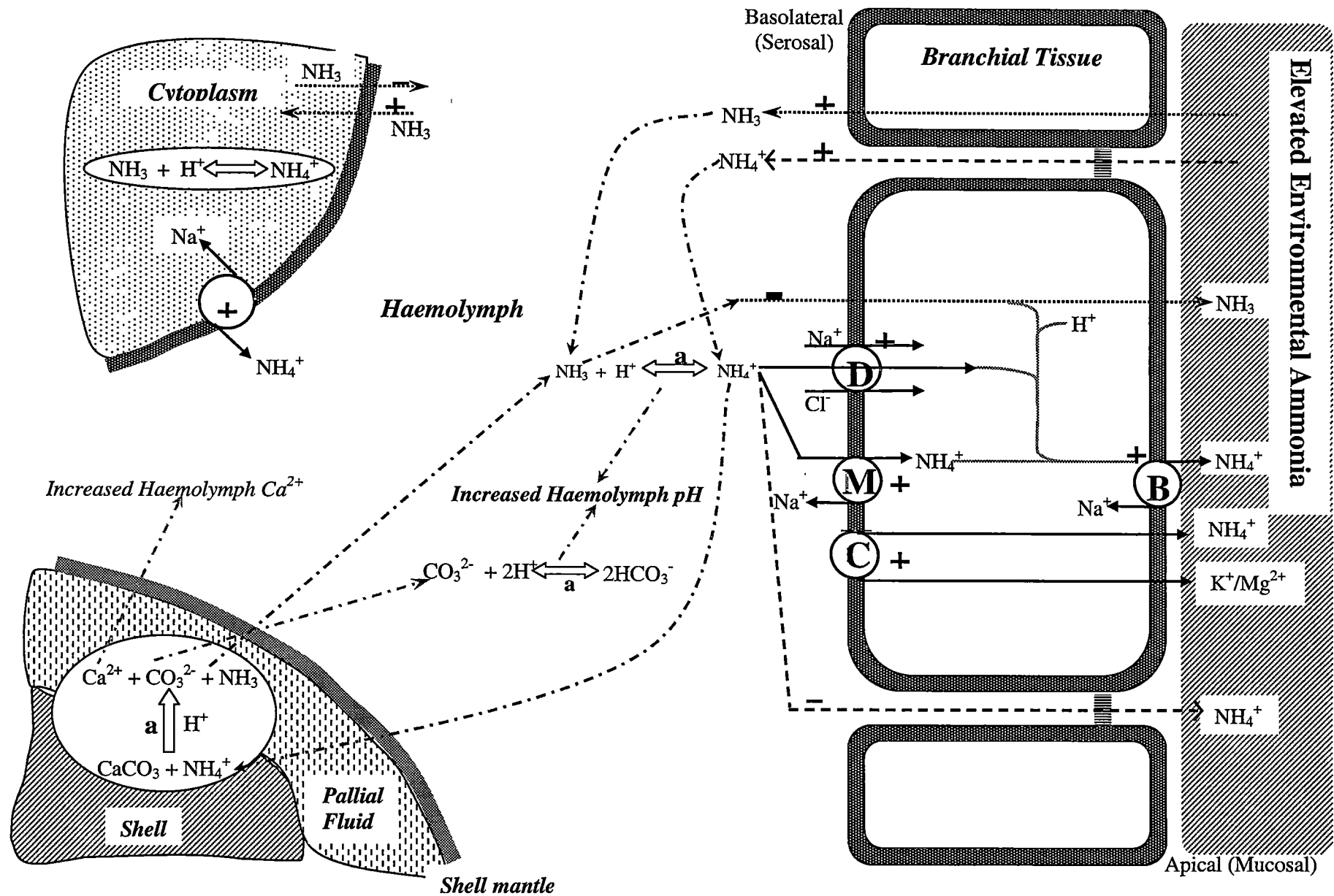










Figure 4.7 Changes in ion exchange and haemolymph chemistry as a result of exposure to elevated environmental ammonia in blacklip and greenlip abalone, based on available literature and results from this study. Ammonia will tend to diffuse into the abalone and diffusion to the external environment will decrease. The net change will depend on the external ammonia level, and normal haemolymph ammonia level for abalone. Active transport is now the main route of ammonia excretion.

# Key

	Lipid bilayer cell membrane
	Diffusion through leaky paracellular junction
	Diffusion through cell membrane
	Active ion exchange
	Leaky paracellular junction
	Increased as a result of ammonia exposure
	Decreased as a result of ammonia exposure
	Likely direction of movement of various ions
<b>a</b>	Altered equilibrium position due to increase in internal ammonia level resulting from elevated environmental levels
<b>M</b>	Basolateral transport mechanism known to be present in vertebrates & invertebrates including molluscs
<b>B</b>	Possible apical transport mechanism, which is known to exist in salmonids, but not shown in invertebrates as yet
<b>C</b>	Possible co-transport mechanism, which may be operating in abalone; similar mechanisms known in salmonids
<b>D</b>	Basolateral co-transport mechanism known to be present in vertebrates, but not yet shown in invertebrates

## CHAPTER 5

### Stocking Density And Refuge Provision In Relation To Growth And Water Quality

#### 5.1 Introduction

As one of the prime determinants of commercial viability in intensive aquaculture systems the relationship between stocking density, stock health and biomass production for a wide range of both vertebrate and invertebrate species has received considerable attention in the literature (Kushnirov & Degani 1991; Allan & Maguire 1992; Chaitanawisuti & Kritsanapuntu 1997; Wagner *et al.* 1997; Hossain *et al.* 1998; Verhoef & Austin 1999). The stocking density that will allow optimal growth rates for a given species is a function of the ecology and behaviour of that species.

Declining growth rates and higher mortality with increasing density is well known in wild populations of herbivorous gastropod molluscs (Creese & Underwood 1982; Fletcher & Creese 1985; Ortega 1985; Stoner 1989) including abalone (Shepherd 1988), which is generally considered a consequence of intra-specific competition for food resources. Declining growth rates with increasing density have also been reported in cultured abalone populations (Mgaya & Mercer 1995; Capinpin *et al.* 1999). As density increases in the confines of a culture system and the submerged surface area remains constant, it is conceivable that competition for food items may make it difficult for individual abalone to gain access to food items thus reducing energy intake. This may increase the duration of foraging excursions, which would incur a substantial energetic cost given the requirement for mucus in gastropod locomotion (Denny 1980), which has been estimated at up to 30% of the energy expenditure for abalone (Peck *et al.* 1987; Donovan & Carefoot 1998). Other density related factors such as an increase in interactions between individuals or competition for

primary attachment area may also act as stressors which divert energy from tissue growth.

Greenlip and blacklip abalone are known to be cryptic up to 80-100 mm, taking refuge in caves and crevices on the reefs they inhabit during the day (Shepherd 1973). Abalone density in localised areas is known to be a function of the total refuge area available, and the refuge area not already occupied (Shepherd 1986a; Shepherd & Partington 1995). The preferred refuge area in greenlip and blacklip abalone with a shell length of 20-100mm consists of crevices in rock formations or between boulders (Shepherd 1973). Douros (1987) observed that when the area available for individual abalone to attach directly to the substrate during the day (defined as the primary attachment area) becomes limited abalone will start to attach either partially or completely to the shells of con-specifics (defined as stacking).

In the artificial conditions of intensive culture systems, factors such as competition for primary attachment area may impose a stress which in the wild would be overcome by migration to less populated areas. Such migration patterns have been documented in wild greenlip abalone populations (Shepherd 1986a; Shepherd & Partington 1995). Constrained within a culture system, an increase in abalone density leads to an increase in stacking and crowding (Mgaya & Mercer 1995; Capinpin *et al.* 1999), which may make it difficult for abalone to emerge from the quiescent daytime phase and commence foraging or even to forage effectively. Increasing the area available for primary attachment at a given density by increasing the level of refuge provision could conceivably alleviate any stress that such stacking and crowding may impose, potentially improving growth rates, stock health and biomass production for a given system.

However, refuges may also interfere with water flow dynamics leading to the development of mini-environments higher in metabolites such as ammonia and lower in dissolved oxygen than the bulk water. Leaching of nutrients may also be affected as water currents are increased or decreased by the presence or absence of refuges. Refuges may also contribute to the accumulation of wastes over a cleaning cycle, and make it more difficult to remove organic wastes when the tanks are cleaned. High stocking density may also influence growth through impacts on water quality as a result of increased oxygen consumption, metabolite

production and accumulation of organic wastes. In order to optimize biomass production the aquaculturist is required to maintain optimal health and growth rates at stocking densities much higher than those likely to be encountered in wild stocks. It is therefore important to understand how factors such as provision of refuges and stocking density interact with biomass production, water quality and stock health in intensive culture systems.

Given the increasing importance of cultured abalone in world markets (Jarayabhand & Paphavasit 1996; Gordon & Cook 2001), the issue of abalone density in culture systems is also starting to receive more attention (Mgaya & Mercer 1995; Marsden & Williams 1996; Capinpin *et al.* 1999). The primary aims of this trial were to investigate the effect of stocking density on growth for greenlip abalone, and the potential to improve growth rates by increasing the level of refuge provision (from 0 to either 1 refuge per 60 abalone or 1 per 30 abalone) at each density (120, 240 and 360 abalone/tank). The impact of stocking density and refuge provision on water quality, both in the water column (bulk water) and under refuges was also investigated. A large amount of data on abalone behaviour was generated from this trial and is presented in Chapter 6.

## 5.2 Methods and Materials

### 5.2.1 Experimental system

The trial was conducted at the same facility described in Chapter 2. The experimental system was based on circular, fibreglass tanks holding 220 L. with central outlets and aerated with three air-stones per tank. The system of 18 tanks was located outside, arranged in three rows of 6, with individual black plastic covers fitted to prevent the formation of algal biofilms. Covers were briefly removed for feeding, tank cleaning and daily observation. Influent oceanic seawater was drawn from sub-surface intakes on an exposed coastline, and was supplied to the system at ambient temperature via header tanks and a 130  $\mu\text{m}$  disc-filter, which was back-flushed daily. Influent water flow was set at 1.8  $\text{L}\cdot\text{min}^{-1}$  per 120 abalone, with submersible aquarium pumps attached to the side of each tank used to standardise the total internal water flow in each tank to 12  $\text{L}\cdot\text{min}^{-1}$  (Table 5.1). A constant total water flow was maintained between density

treatments to prevent the different influent water flow rates from confounding consumption or foraging activity (Higham 1998).

### ***5.2.2 Experimental protocol***

The basis of the treatments, shown in Table 5.1 and Figure 5.1, was a factorial design of three stocking densities (low, medium, high with 120, 240, and 360 abalone/tank respectively) with three levels of refuge provision at each density (0, 1/60 and 1/30 abalone). Lengthwise half-sections of 150 mm PVC (280 mm long) pipe were used for the refuges. The refuges were arranged symmetrically in each tank, with equal numbers perpendicular and parallel to the clockwise, circular water current (see Chapter 6, Plates 6.5, 6.11, 6.17). Polypropylene rings filled with sand attached to the top of the refuges prevented movement from the designated position. Abalone density ranged from 62-212 abalone.m<sup>-2</sup> submerged surface area (SSA), and the abalone initially covered from 12-40% of the available surface area.

### ***5.2.3 Surface Area Calculations***

The submerged surface area (SSA) was defined as the total surface area that was submerged and available for attachment during the daytime quiescent period, and consisted of the walls, floor and curved surface area of the refuges. In determining the curved surface area of the refuge, it was assumed that only one side of the half section of pipe was available to the abalone as observations indicated that the abalone tended to use only the one side during the day (see Chapter 6) . In calculating SSA water depth at the outer wall of the tank was used, and the curved surface area of the refuges was based on average measurements from 24 refuges. Effective foraging area (EFA) was defined as the flat area on the bottom of the tank where food items would be encountered, and was calculated as the bottom surface area of the tank minus the flat area covered by the refuges.

Photocopied images of 150 abalone were used to determine the portion of a paper disc with a diameter equal to the shell length of the abalone that was actually covered by that abalone, by weighing the whole disk and then the portion of the disk actually covered by the abalone shell. Using the average shell

length of the abalone in a tank the average surface area of individual abalone was determined and thus the total surface area of abalone in each tank could be calculated. In calculating the percentage cover by abalone, it was assumed that each abalone was attached directly to the available substrate and there was no stacking.

#### ***5.2.4 Experimental animals***

The experimental abalone were cultured greenlip abalone, about 4 years old at the start of the trial, and the growth trial was conducted from December 1997-April 1998. Prior to the start of the experiment they had been held in a single large holding tank for several months, being fed a combination of algal and formulated diets. The experimental tanks were stocked at the experimental density for 2-3 weeks before the abalone were weighed and measured for the start of the growth trial. This involved anaesthetizing the abalone with benzocaine ( $1 \text{ mL.L}^{-1}$  of a 10% stock solution in ethanol (w/v)), and tagging 120 animals from each tank with polyethylene tags (Hallprint, Adelaide) attached with cyano-acrylate adhesive gel. In the medium and high density treatments (240 & 360 abalone/tank) the abalone were randomly selected (in groups of 20) for tagging, and the remainder exposed to air for the same time (15-20 minutes). These animals were weighed (to 0.01 g) and measured for length (to 0.1 mm) (average initial length  $57.3 \pm 0.5 \text{ mm}$ , average initial weight  $24.61 \pm 0.91 \text{ g}$  (tank means  $\pm$  SD,  $n=4500$ )). At the end of the growth trial, the tagged animals were weighed and measured for calculation of individual growth rates, which were averaged to provide a tank mean for statistical analysis. Growth indices calculated are given in Chapters 3 (Section 3.3.3)

Initial biomass was calculated using the average weight of the tagged abalone in each tank, multiplied by the total number of abalone added at the start of the trial.

#### ***5.2.5 Food Conversion Ratio***

The daily ration was weighed out for each tank, and adjusted such that 5-10% was remaining the following morning. A commercial FCR was determined by using these data to calculate the total amount of food fed to each tank over the



trial, and dividing this by the gain in biomass for that tank (kg food fed.kg biomass gain<sup>-1</sup>). No allowance was made for uneaten food, or moisture content of the diet. The biomass gain was determined by subtracting the final biomass from the initial biomass. Initial and final biomass was calculated as a product of the average weight of tagged abalone at the start and end of the growth trial and the number of abalone in that tank (corrected for mortalities at the end of the growth trial).

#### ***5.2.6 Tank maintenance***

The abalone were fed daily, between 13.00-17.00, with feeding rates adjusted on demand for each tank. The average daily feeding rate for the trial is shown in Table 5.1. The most recent formulation of a proprietary formulation (ABCHOW) was used, being extruded through an industrial pasta-extruder, dried for 40-50 °C for 24-48 hours and stored at ambient temperature until required. Fresh diet was made up on site as required.

The tanks were cleaned every 4 days by draining the water through the central outlet and hosing out the wastes with ambient seawater, with the shelters being carefully raised just enough to allow accumulated wastes to be hosed out. The tanks with 6, 8 or 12 shelters took slightly longer to clean, but no tank was empty longer than 3 minutes before refilling commenced. The tanks were rapidly refilled with ambient seawater such that 95% of the animals in each tank, were submerged within 8-10 minutes of the tank being emptied. Cleaning was postponed at times due to unfavourable weather conditions (rain, excessive wind or elevated air temperature), in which case feeding was also suspended.

#### ***5.2.7 Water quality***

Temperature and dissolved oxygen were monitored routinely in the morning (generally between 08.00-10.00) and afternoon (between 15.00-17.00). Ambient bulk-water quality (ammonia, nitrite, pH, DO, temperature) was measured on average every 10 days between 10.00-12.00, with samples for ammonia and nitrite collected in acid-washed sample bottles. Water quality under the refuges was measured as described below.

Samples for measuring water quality under the hides were collected with a plastic 50 mL syringe (acid -washed, rinsed in de-ionised water), with a 150 mm length of 4 mm polypropylene tubing used to ensure that the centre of each refuge was always sampled. Care was taken to minimise disturbance of the environment under the hide during insertion and removal of the tubing, in order to collect a representative sample. A small volume of tank water was used to rinse the syringe before collecting the sample, which allowed a bubble-free sample to be taken. Samples contaminated either with sediment or air were rejected, and that hide was not re-sampled on that day. Bulk water was also sampled in this manner at the same time to allow a direct comparison. Aeration was maintained as normal in tanks during sampling.

On removing the syringe from the water, the 4 mm tubing was crimped to seal the sample. Ten to twelve samples were collected at one time, and were kept in the shade until taken inside for analysis. DO and then pH were recorded within 5 minutes of the sample being drawn, with the probes being placed inside the syringe barrel after carefully removing the plunger. The samples were then filtered through GF/C filters for analysis of total ammonia.

Total ammonia (TA) was measured using the phenol method of Solorzano (1969). Unionised ammonia (UIA) was calculated according to the equation in Bower & Bidwell (1978) from TA, using temperature and pH. Sand-filtered ambient seawater was used for making all standards. DO was measured using a TPS WP-82Y meter in conjunction with a YSI probe or WTW Oxi-96 meter and probe. The probes were checked against saturated seawater or Winkler titrations. Temperature was measured using the thermistor on the DO probes, which was checked against a mercury thermometer calibrated to  $\pm 0.01$  °C. pH was measured using TPS WP-81 probe and meter, which was calibrated prior to use in fresh buffers at pH 7 and 9.28 (Bruno & Svoronos 1989).

### ***5.2.8 Effect of Current Speed on Diet Composition***

Three of the tanks used in the growth trial were subsequently set up to assess the effect of current speed on diet composition. Influent water flow was set at 200-300 mL.min<sup>-1</sup> for each tank, with the water inlet angled such that influent water did not contribute to the internal water current. The

submersible aquarium pumps used in the growth trial were used to set the water flow to 6 or 12 L.min<sup>-1</sup>, with a third tank acting as a control with no added internal circular water current but the same rate of water exchange. Samples of diet were weighed onto pre-weighed screens and submerged for 24 hours, with 4 trays at each current speed. The trays were removed, drained of excess water, frozen and subsequently dried at 80°C for 48-96 hours before being weighed. Samples from 2 of the 4 trays were ground to a fine powder and analysed for carbon and nitrogen in an elemental analyser. The dry matter loss at each current speed was determined using a correction for moisture content of the unleached diet.

#### ***5.2.9 Statistical analysis***

All data were checked for homogeneity of variance and normality by observation of residual and distribution plots generated by JMP 3.2. No transformations were necessary for any data analysed.

Using SPSS 10.02 (SPSS Inc.) analysis of variance was used to detect significant treatment effects, with Tukeys HSD used to compare individual means. Two-way analysis of variance was used to check for a significant interaction between stocking density and level of refuge provision in terms of the growth indices measured. T-tests were also used to specifically compare results for growth, FCR and tag loss from treatments with no refuges (120/0, 240/0 & 360/0) with the results from 1 refuge per 30 abalone (120/4, 240,8 & 360/12). The data from the ANOVA are given in Table 5.3 (F, degrees of freedom, P), and in Table 5.4 for the t-tests.

### **5.3 Results**

While there was some movement of the shelters, the polypropylene rings prevented the shelters moving to the center of the tank, and kept them in the same relative position for each treatment. Within 3 weeks of commencing the growth trial, spawning activity in all tanks was either directly observed or inferred from the presence of gametes in tanks.

### **5.3.1 Mortality**

Mortality was less than 3.5% for any one tank, and averaged 0.6% over the whole trial.

### **5.3.2 Water quality**

Figure 5.2 shows the ambient bulk-water quality data for pH, TA and UIA throughout the growth trial. Although no significant effects could be detected when comparing individual treatments ( $P>0.05$ ), pooling the refuge treatments at each density revealed significant density-related effects on bulk water quality ( $n=6$ ) even though the influent water flow was set at  $1.2 \text{ L}\cdot\text{min}^{-1}$  per 120 abalone. The lowest stocking density had significantly higher dissolved oxygen levels than high density tanks (4.642, 2, 15,  $P=0.027$ ), and high density tanks had significantly higher levels of TA than the other two treatments (4.659, 2, 15,  $P=0.027$ ).

Comparing ambient bulk-water quality with water quality under the refuges showed that DO tended to be lower, and TA tended to be higher under the refuges compared to the bulk water but there were no significant differences between the two environments ( $P>0.1$ ).

### **5.3.3 Growth Data**

There was a degree of tag loss for each treatment, which precluded growth data being generated for every individual measured at the start of the growth trial. A 2-way ANOVA did not reveal a significant interaction between the level of refuge provision and stocking density for either SGR-L or SGR-W, although density was a highly significant effect for both indices ( $P<0.001$ , Table 5.3). There was a significant interaction in between density and refuge provision in terms of  $\mu\text{m}\cdot\text{d}^{-1}$  (3.673, 4,  $P<0.001$ ). Growth data for individual treatments in terms of length (SGR-L) and weight (SGR-W) is given in Figure 5.3. In terms of SGR-W, abalone from 120/0 treatment grew significantly faster than abalone from the 240/0 and 360/6 (1 refuge per 60 abalone) treatments (4.931, 8, 9,  $P=0.014$ ). In terms of length (SGR-L &  $\mu\text{m}\cdot\text{d}^{-1}$ ), growth rates were significantly higher in the 120/0 treatment compared to abalone in the 240/0, 240/4 (1 refuge per 60 abalone), 360/0 and 360/6 treatments (SGR-L – 13.929, 8, 9,  $P<0.001$ ;

um.d<sup>-1</sup> – 8.273, 8, 9, P=0.002). At low density, growth rates declined as the level of refuge provision increased from 0 to 1 per 30 abalone, with t-tests showing this to be significant (P<0.1, Table 5.4, Figure 5.3). At medium and high densities growth rates improved as the level of refuge provision increased, and t-tests showed that growth was significantly higher from the high density treatment in terms of both length and weight with 1 refuge/30 abalone compared to no refuges (P<0.1, Table 5.4, Figure 5.3).

There were no significant differences in length:weight ratios, or length gain:weight gain between treatments (P>0.05) (data not shown).

Figure 5.4 shows the growth data in terms of abalone per unit area of submerged surface area. Growth increased at both medium and high densities with an increase in the level of refuge provision and the resulting increase in SSA. Figure 5.6 shows that although EFA was reduced as the level of refuge provision increased at each density, growth rates increased at medium and high densities, but not at low density. This was particularly evident for length.

### ***5.3.5 Consumption and Food Conversion Ratio***

Daily ration ranged from 0.62-0.73 % biomass.day<sup>-1</sup>, and did not differ significantly between treatments (Table 5.1). The FCR data were calculated as commercial FCR, with no allowance for uneaten food, but the daily ration per tank was adjusted for each individual tank. When pooled for density, FCR was significantly lower in the low density treatments compared to the medium and high density treatments, which did not differ significantly from one another (Figure 5.6, Table 5.3).

There were significant treatment effects (120/0 being significantly lower than the FCR from 240/0 and 360/6, Table 5.3, Figure 5.7) (4.163, 8, 9, P=0.024). Based on a t-test, FCR was significantly lower at 360/12 (1 refuge per 30 abalone) compared to 360/0 (no refuges) (t=4.133, 2, p=0.054) (Table 5.4, Figure 5.7).

### ***5.3.6 Effect of current speed on diet composition***

There were no significant changes in diet composition after food pellets were exposed for 24 h to current speeds of 0, 6 or 12 L min<sup>-1</sup> (P>0.05) (Table 5.2).

### **5.3.7 Economic Projections**

Economic projections were developed using the biomass gain data, and incorporating the major running costs of land-based abalone farming (labour, water and food). The cost of production per kg biomass gain was lower at the highest density (\$A 51), compared to medium and low densities which were similar (\$A 63 & 67) (Figure 5.8). Water exchange was a major part of the total cost of production.

## **5.4 Discussion**

Mortality was low throughout the trial, even in treatments stocked at twice the commercial stocking rate (40 kg.m<sup>-3</sup>). Other authors also report no increase in mortality for abalone held at high stocking densities (Mgaya & Mercer 1995; Marsden & Williams 1996; Capinpin *et al.* 1999). Neither has mortality been reported for other benthic invertebrate species at high stocking densities (spotted babylon, Chaitanawisuti & Kritsanapuntu 1997; yabbies (*Cherax destructor*), Verhoef & Austin 1999; penaeid prawns, Allan & Maguire 1992). The mortality data indicate that, given sufficient water exchange, higher than normal stocking densities will not necessarily adversely affect stock health in the short term. However, it is likely that tanks at the highest stocking density are approaching or exceeding the capacity of the system to maintain stock health, and compared to the lower density treatments would be at a higher risk of mortality or infections from stress related factors either directly or following a stress event such as systems failure. While an increase in mortality has been reported in wild populations of inter-tidal limpets as density increased and food became limiting (Creese & Underwood 1982; Ortega 1985), the use of nutrient dense formulated diets would prevent food becoming a limiting factor for cultured gastropods. In the current trial it would be expected that the use of a formulated diet, with ration adjusted on a per tank basis, would prevent food becoming a limiting factor on its own, and other density related effects would then impact on growth.

Greenlip abalone are synchronous spawners that normally aggregate for spawning. Reproductive maturation and spawning for wild stocks of this species starts at 80-100 mm shell length (Shepherd & Laws 1974; Shepherd 1986b). However, personal observation of this species and anecdotal evidence indicate that cultured stock being fed on formulated diets can mature and spawn at 50-60 mm. Although handling stress may have been a factor in the spawning event observed at the start of this trial, it also coincided with the natural spawning season for this species at this site. While spawning was observed in all tanks, it can not be ascertained if all animals in each tank spawned, which may have contributed to the variation in growth data. Gonad development requires a substantial energy investment, and growth in wild abalone stocks has been shown to be reduced during the spawning season (Shepherd & Hearn 1983). Abalone which have spawned may lose up to 10% of the live weight (Gabbott 1983; Carefoot *et al.* 1998).

Given that the difference in influent water flows between density treatments could lead to a difference in water currents between density treatments, submersible aquarium pumps were used to standardise water currents as it has been shown that water velocity can influence food consumption and growth in greenlip abalone (Higham 1998). While the submersible pumps generated water currents in the outer areas of the tank, the presence of refuges did reduce water currents in the central area of the tank (see Chapter 6 for details). This gave rise to the potential for differences in water currents to impact on leaching of nutrients from the immersed diet before it was grazed by the abalone, especially in tanks with no refuges compared to tanks with refuges. However, no effect of water current was detected on diets immersed for 24 hours (Table 5.2).

It is possible that the statistical power of the growth trial may have been at least partly reduced by the fact that the experimental abalone were derived from the slower growing component of a cohort. At 4 years of age, cultured greenlip abalone are more likely to be >80mm, rather than an average of 60mm. However there was little choice in the stock available for the trial, and significant effects on growth in terms of both length and weight were still detected. Running the growth trial for longer may have allowed treatment effects to

become more pronounced, but water temperatures were declining with the onset of autumn. It is unavoidable that at times the ideal experimental design is constrained by practical issues such as what stock is available and ambient water temperature and the results need to be interpreted in light of these limitations (Tacha *et al.* 1982).

While the growth rates given in Figure 5.3 are generally lower than commercial growth rates for abalone in Australia (80-100  $\mu\text{m}\cdot\text{day}^{-1}$ ), they are within the range quoted in the literature for cultured abalone on formulated diets (reviewed in Fleming *et al.* 1996; Coote *et al.* 1996; Sales & Britz 2001), and significant effects on growth rates were still detected in terms of both length and weight. The effect on growth in terms of weight was not as marked as for growth in terms of length, which may be at least partly due to the spawning event discussed above. It is also possible that the growth response in terms of length and weight differed, with soft tissue growth being more sensitive than shell growth to crowding and competition for primary attachment space as density increases. It is known that shell growth and soft tissue growth are not directly linked in gastropods (Palmer 1980; Harris 1999).

Analysis of the growth data showed that density was a significant factor influencing growth in this trial, with both SGR-L and SGR-W declining as density increased. Mgaya & Mercer (1999), in a study of the ormer (*H. tuberculata*) using juveniles up to 23 mm and densities of 500-2000  $\text{m}^{-2}$ , also showed the lowest growth at the highest density, and suggested that the effect of density is a consequence of competition for food and primary attachment area. Growth in terms of both length and weight also declined in paua (*H. iris*) as density was increased, despite no change in food consumption between low and high density treatments (up to 40 abalone. $\text{m}^{-2}$  SSA at 35-40 mm) (Marsden & Williams 1996). A decrease in growth rates with an increase in density means less energy was available for growth, suggesting that either food consumption was reduced or that conversion of ingested ration to biomass was less efficient at higher densities. While Table 5.1 shows that ration provided (which was adjusted on a per tank basis over the course of the trial such that 5-10% was left uneaten) did not differ between densities, Figure 5.6 shows that FCR was significantly higher at medium and high densities (when refuge provision



treatments were pooled together). This further supports the suggestion that ration was less efficiently converted to biomass as density was increased. The significant increase in tag loss with density (Figure 5.7) also supports the notion of an increase in interaction between individuals, which in turn could lead to increased stress, a higher FCR and reduced growth rates.

Provision of refuges increased growth rates at medium and high densities, with a significant interaction between refuge provision and density in terms of  $\mu\text{m.d}^{-1}$ . This increase in growth from 0 refuges to 1 refuge/30 abalone was significant at high density (based on t-tests) (Figure 5.4). If growth is depressed by increasing interaction among con-specifics as density increases as outlined above, then it would be expected that increasing the submerged surface area (SSA) would reduce these interactions and improve growth rates. Figures 5.1 & 6.1 clearly show the effect of increasing the level of refuge provision on coverage of the SSA. The decline in tag loss as the level of refuge provision increases (and hence SSA increases) at medium and high densities is clear evidence of a decreasing interaction between individuals (Figure 5.7), either during the quiescent phase or while actively foraging. A reduction in crowding under the refuges would also make it easier for individual abalone to leave the resting spot used for the quiescent period and commence active foraging.

Observations from this trial indicate that only a percentage of abalone are active for any given foraging period (see Chapter 6), which is well established in populations of both wild herbivorous gastropods (Hartnoll & Wright 1977; Little & Stirling 1985; Evans & Williams 1991) and cultured abalone (Uki 1981; Knauer *et al.* 1995; Donovan & Carefoot 1998; Nakamura & Archdale 2001). In a study of South African abalone (*H. midae*), Knauer *et al.* (1995) reported that in a given tank only 30-40% of the abalone were active on any one night, with only 5-10% actively grazing on the ration provided at any one time. In the former, Donovan & Carefoot (2001) noted five different levels of activity from fully quiescent to actively grazing, with some individuals exhibiting different activity states on some nights but not actually foraging. In the high density treatments abalone not actively foraging would be more disturbed than abalone at lower density, which could interfere with the biological function that these different activity levels would have.

The decrease in effective foraging area observed in this trial as a result of using curved refuges (Figure 5.1, 6.1) would actually increase the effectiveness of foraging activity by increasing the chance of an abalone finding food. In practical terms this amounts to an increase in food abundance, which has been shown to improve growth in abalone (Tahil & Juinio-Menez 1999). Observations from the current study (presented in Chapter 6) and other studies (Donovan & Carefoot 1998; Nakamura & Archdale 2001) show that foraging abalone tend to follow a random path in the search for food items, grazing briefly on any food they encounter before moving on. Based on evidence from wild populations of other herbivorous gastropods (Chelazzi *et al.* 1993; Della Santina *et al.* 1995), it would be expected that abalone will stop foraging once the crop is full and then return to a suitable resting spot for the quiescent period. If the crop is filled quicker because of greater food abundance, this would reduce the energy expenditure on locomotion. In gastropods movement is energetically expensive because of the mucous required (Denny 1980; reviewed Donovan & Carefoot 2001), which has been shown in different studies to require up to 30% of the overall energy budget (Peck *et al.* 1987; Donovan & Carefoot 2001). Reducing the energy required for foraging activity and thus mucous production would allow more energy to be available for growth. This may explain the decrease in FCR at medium and high density as the level of refuge provision increased from 0 to 1/30 abalone, which was significant at high density (Figure 5.7), which indicates that more energy from the ingested ration is available for somatic growth.

At low density (120 abalone per tank), growth declined as the level of refuge provision increased (Figure 5.3), which differed from the pattern observed at medium and high density (240 & 360 abalone per tank). This may be due to an absence of density effects at the low density used in this trial, and the absence of refuges may be beneficial in this case. At medium and high densities the increased competition for resting and foraging areas allows the effect of refuge provision on growth to become more pronounced. The use of round refuges means that the decrease in EFA is linked directly to the increase in SSA, and the importance of one effect over the other cannot be quantified. Such a

determination would require the use of flat topped refuges as well, which would increase the level of refuge provision but affect EFA.

While there were no significant differences in ambient bulk-water quality between individual treatments, pooling the data for stocking density revealed that DO was significantly higher and TA significantly lower at low density compared to high density (Figure 5.2). Although statistically significant, the differences observed were small and, based on chronic bioassay data, unlikely to cause growth reductions either alone (Harris *et al.* 1998a; Harris *et al.* 1999a) or in combination (Chapter 2, Chapter 3). These results show that elevated ammonia and low dissolved oxygen can occur together in abalone systems, which is not unexpected in intensive aquaculture (Millamena 1990; Allan *et al.* 1995). The effect of density on water quality suggests that the influent water per abalone (nominally 1.8 L.min<sup>-1</sup> per 120 abalone) was insufficient for the highest biomass. At high stocking densities, more wastes will accumulate and they will degrade faster (Allan *et al.* 1995), and these wastes have the potential to consume oxygen and produce ammonia (investigated in Chapter 7).

No difference was found between the bulk water quality and water quality under the refuges, in terms of dissolved oxygen, ammonia (either total or un-ionised) or pH, which indicates that there was good exchange between these environments. The lack of any difference shows that even at the highest density with the least number of shelters, water quality under the refuges did not deteriorate to the point that abalone growth or health could be affected. However, substantial amounts of organic wastes did accumulate over the cleaning cycle, which have the potential to impact on water quality or stock health should these wastes not be removed during cleaning. The influence of these wastes on water quality is investigated in Chapter 7. It should be noted that DO was lower and TA was higher under the refuges compared to the bulk water, even though the differences were not significant. Should water exchange be interrupted or reduced, then this environment could become degraded to the point that growth rates or stock health is compromised.

Some basic economic projections were developed based on the growth data which compared the costs of production and anticipated returns at the different densities. Although not intended to be a detailed economic analysis of abalone

culture, these projections demonstrate that the most cost effective biomass production was achieved at the highest stocking density, even though this density resulted in the lowest overall growth rate. Faster overall growth rates are therefore not the only consideration in determining commercial stocking densities, as the highest biomass production may not be achieved by the highest growth rates. This has also been shown for penaeid prawns (Maguire & Leedow 1983; Allan & Maguire 1992)

The results presented in this chapter show that while stocking density did have significant effects on water quality, water exchange also had an effect, and possibly the accumulation of organic wastes. The impact of water exchange and organic wastes on bulk water quality is further explored in Chapters 7 & 8. In the next chapter, observations and behavioural data are presented which further clarify the mechanism which reduces growth as stocking density increases, and the effect of refuge provision on this mechanism.

### **5.5 Conclusion**

Overall growth rates were higher at low density compared to medium and high stocking densities, with evidence from FCR and tag loss data that this was related to an increase in con-specific interactions. There was a significant interaction between density and level of refuge provision in terms of microns per day, and growth rates were improved by adding refuges at medium and high density (significant for high density). This improvement is due to a number of factors, including the increase in submerged surface area reducing con-specific interactions and a reduction in effective foraging area effectively increasing food abundance and improving the efficiency of foraging.

Water quality under refuges did not differ significantly from the bulk water, although DO was lower and TA was higher, and this could affect stock health should water exchange be inadequate. DO was significantly lower and TA significantly higher at high compared to low stocking density.

Treatment	Refuge Provision	Initial Biomass (kg.m <sup>-3</sup> )	% Cover of SSA (initial)	% Cover of SSA (final)	Average Ration Provided (% biomass)	Food Conversion Ratio	Influent Water Flow (L.min <sup>-1</sup> )	Pump Water Flow (L.min <sup>-1</sup> )
120/0	0	14.12±0.87	12	14	0.62±0.00	1.50±0.11	1.73±0.07	9.93±0.09
120/2	1/60	13.42±0.24	12	16	0.61±0.00	1.56±0.17	1.82±0.01	9.96±0.06
120/4	1/30	13.91±0.51	14	16	0.73±0.00	1.91±0.10	1.69±0.04	9.93±0.17
240/0	0	28.82±0.98	20	24	0.69±0.00	2.42±0.35	3.45±0.01	8.03±0.13
240/4	1/60	27.75±2.29	23	26	0.64±0.00	2.21±0.21	3.47±0.08	8.16±0.08
240/8	1/30	28.23±0.15	27	31	0.66±0.00	2.08±0.16	3.36±0.03	8.15±0.07
360/0	0	40.96±1.56	27	33	0.70±0.00	2.29±0.02	5.13±0.05	6.66±0.15
360/6	1/60	42.33±1.91	32	38	0.63±0.00	2.47±0.11	5.17±0.05	6.72±0.16
360/12	1/30	40.46±1.55	40	46	0.64±0.00	1.93±0.08	5.19±0.01	6.62±0.11

Table 5.1 Summary of experimental design for the growth trial. Values are means for duplicate tanks (n=2, ±SE). Cover of submerged surface area (SSA) was calculated as described in the text (5.2.3). Ration through out the trial was adjusted on a per tank basis such that <10% of the food provided remained the following morning. Influent water flow was nominally set at 1.8 L.min<sup>-1</sup> per 120 abalone, with the submersible aquarium pump adjusted to provide a total nominal flow of 12 L.min<sup>-1</sup> per tank. Treatment is given as abalone per tank/refuges per tank. Refuge provision is number of refuges per abalone in the tank.

	Dry Matter Loss (%)	% Carbon	% Nitrogen	C:N Ratio
Fresh Diet		33.7±1.05	4.41±0.18	7.65±0.07
No Current	20.04±0.39	32.18±1.56	3.94±0.23	8.18±0.08
6 L.min <sup>-1</sup>	20.31±0.22	34.06±0.64	4.07±0.17	8.38±0.19
12 L.min <sup>-1</sup>	20.53±0.49	36.84±1.68	4.47±0.18	8.23±0.03

Table 5.2 Analysis of diets leached at different current speeds for 24 hours. Values are means±SE (n=4 for dry matter loss, n=2 for composition). None of the changes shown were significant (P>0.05).

Variable	n	Factor	F ratio	Degrees of Freedom	P
<b><i>Water Quality</i></b>					
<b><i>Bulk Water</i></b>					
pH	6		1.555	2, 15	0.243
Dissolved Oxygen (mg.L <sup>-1</sup> )	6		4.642	2, 15	0.027
Total Ammonia (mg.L <sup>-1</sup> )	6		4.659	2, 15	0.027
<b><i>Refuges cf. Bulk Water</i></b>					
pH	2		0.468	7, 8	0.833
Dissolved Oxygen (mg.L <sup>-1</sup> )	2		0.194	7, 8	0.978
Total Ammonia (mg.L <sup>-1</sup> )	2		0.907	7, 8	0.545
<b><i>Growth Data</i></b>					
<b><i>2-Way Factorial</i></b>					
$\mu\text{m.day}^{-1}$	2	Density	44.988	2	0.000
		Refuges	3.381	2	0.080
		Interaction	3.673	4	0.049
SGR-L (%.day <sup>-1</sup> )	2	Density	25.441	2	0.000
		Refuges	2.617	2	0.127
		Interaction	2.517	4	0.115
SGR-W (%.day <sup>-1</sup> )	2	Density	15.270	2	0.001
		Refuges	2.275	2	0.159
		Interaction	1.091	4	0.417
<b><i>Treatment Comparisons</i></b>					
$\mu\text{m.day}^{-1}$	2		8.273	8, 9	0.002
SGR-L (%.day <sup>-1</sup> )	2		13.929	8, 9	0.000
SGR-W (%.day <sup>-1</sup> )	2		4.931	8, 9	0.014
<b><i>Food Conversion Ratio</i></b>	2		4.163	8,9	0.024
<b><i>% Tag Loss</i></b>					
Individual Treatments	2		1.876	8, 9	0.184
Pooled for Density	6		5.150	2, 15	0.020

Table 5.3 Data from ANOVA performed on the variables indicated. Tukeys HSD was used as a post-hoc test to compare individual means, and observation of residual plots to assess homogeneity of variance.

	Density	n	t statistic	P
<i>Growth Data</i>				
$\mu\text{m.day}^{-1}$	120	2	5.372	0.033
	240	2	-1.930	0.193
	360	2	-3.280	0.081
<i>SGR-L (%.day<sup>-1</sup>)</i>	120	2	6.168	0.025
	240	2	-1.793	0.215
	360	2	-3.004	0.095
<i>SGR-W (%.day<sup>-1</sup>)</i>	120	2	3.56	0.072
	240	2	-0.553	0.635
	360	2	-3.609	0.069
<i>Food Conversion Ratio</i>	120	2	-2.754	0.110
	240	2	0.896	0.465
	360	2	4.133	0.054

Table 5.4 Data from t-test comparing results from treatments with no refuges with results from provision of 1 refuge per 30 abalone (120/0 & 120/4, 240/0 & 240/8, 360/0 & 360/12). Density is abalone per tank. Tests were performed using Excel XP.



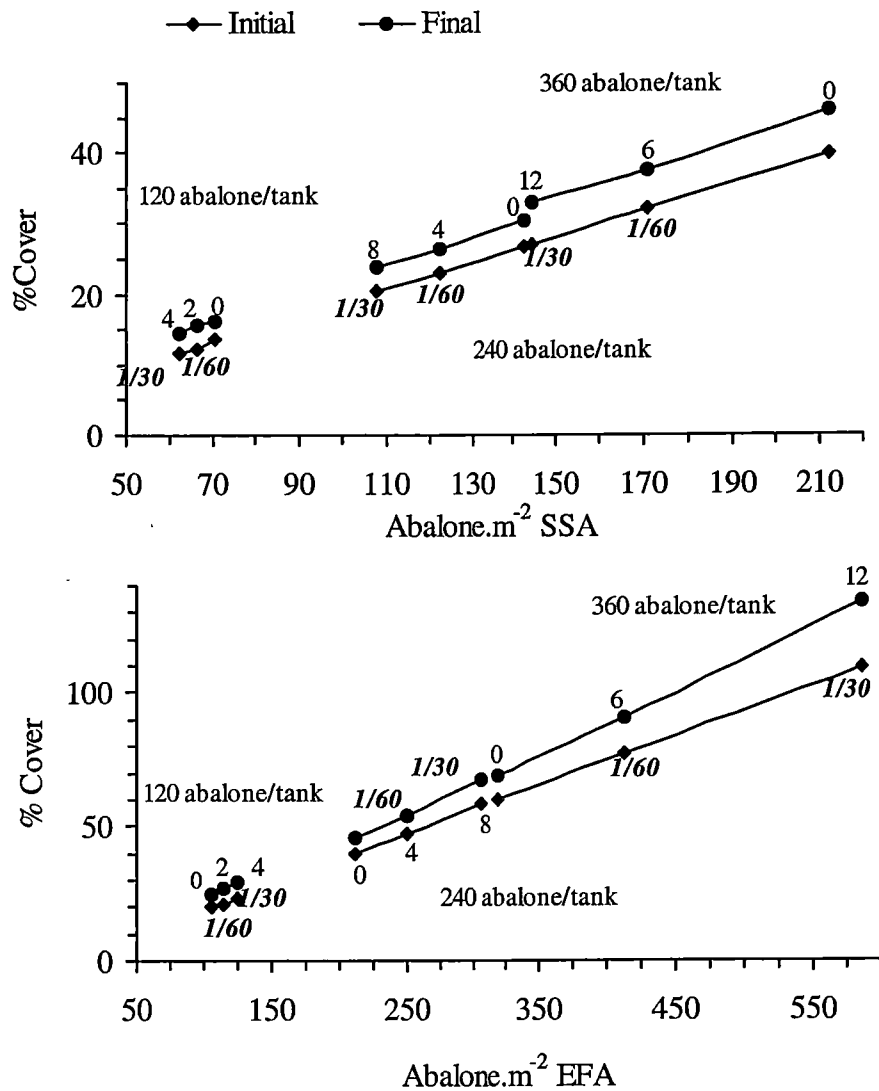


Figure 5.1 Percentage cover of submerged surface area (SSA) and effective foraging area (EFA) by abalone at the start and end of the growth trial in relation to abalone density for each treatment. SSA is the submerged surface area available to the abalone during the quiescent daytime period. EFA is the area where food is available to be grazed. As SSA increases, EFA decreases. Abalone surface area was determined by paper planimetry as described in the text (Chapter 5, 5.2.3). Numbers above the line are refuges per tank; numbers below the line indicate level of refuge provision.

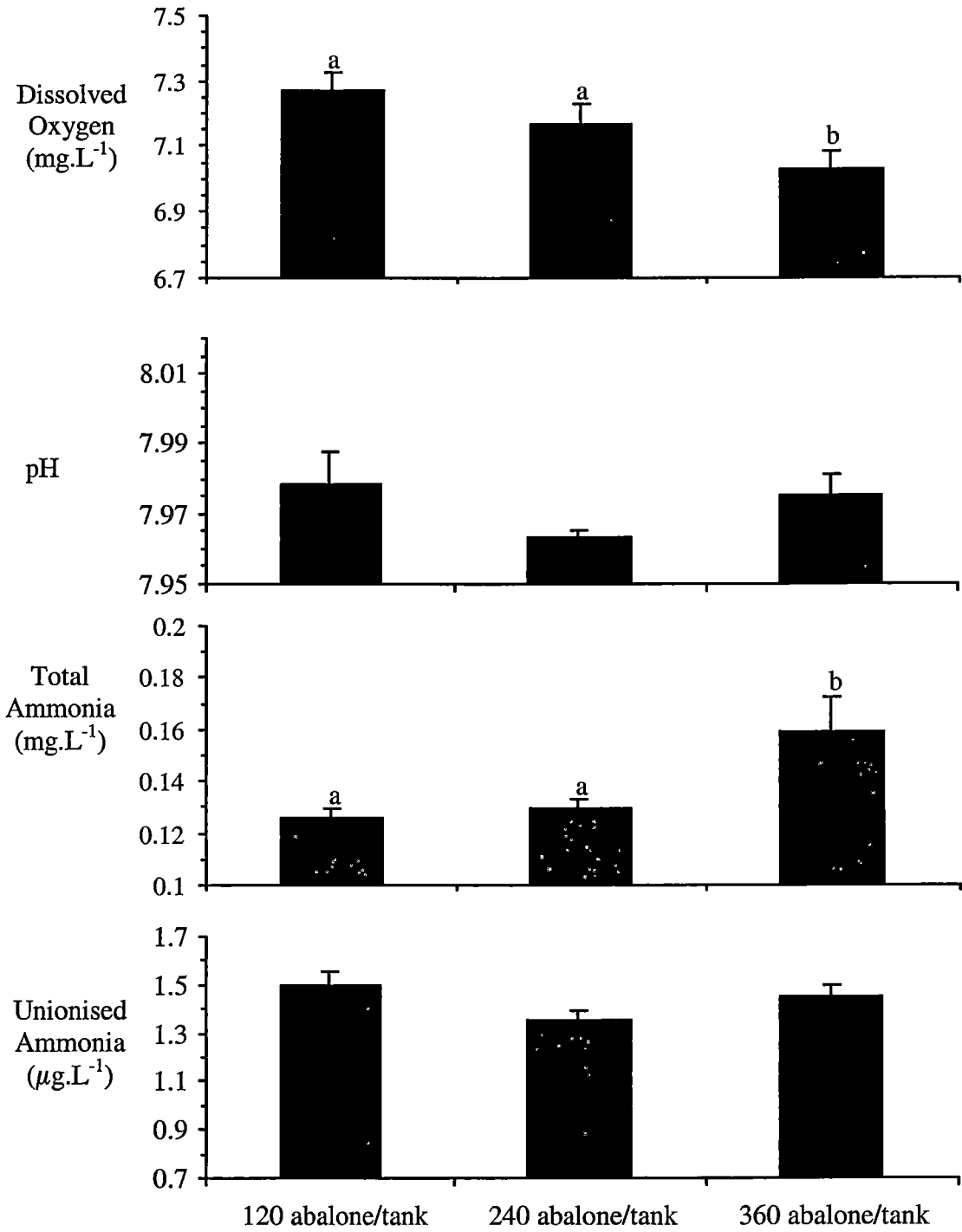


Figure 5.2 Bulk water quality measured during the growth trial (means±SE, n=6) based on sampling every 7-10 days through out the growth trial. Results were averaged for individual tanks, then pooled for density. Different superscripts indicate significantly different means (P<0.05).

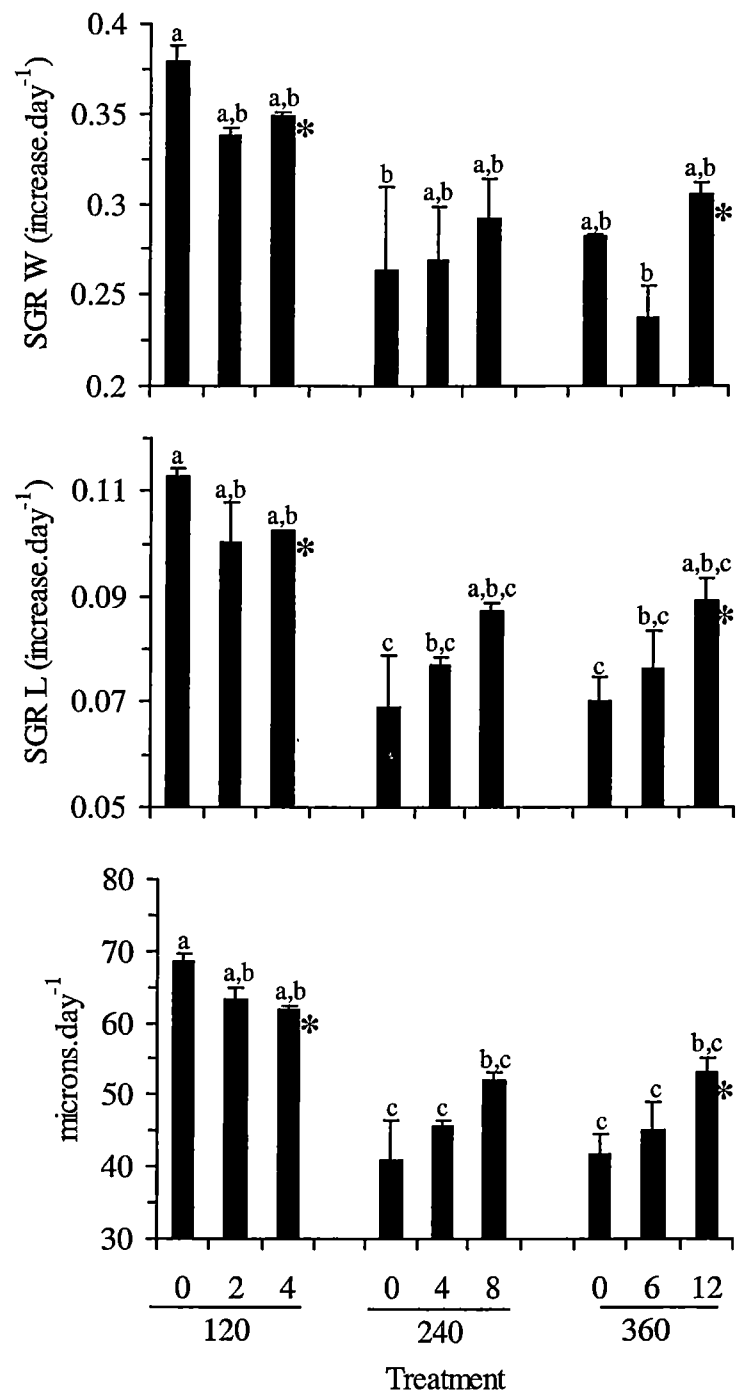


Figure 5.3 Growth data for greenlip abalone from the experimental treatments (means±SE, n=2). Different superscripts indicate significantly different means based on ANOVA ( $P<0.05$ , Table 5.3). The numbers under the columns are the number of refuges per tank, and the number of abalone per tank. An asterisk indicates significant difference between no refuges (120/0 & 360/0) and 1 refuge per 30 abalone (120/4 & 360/12) based on individual t-tests ( $P<0.1$ , see Table 5.4).

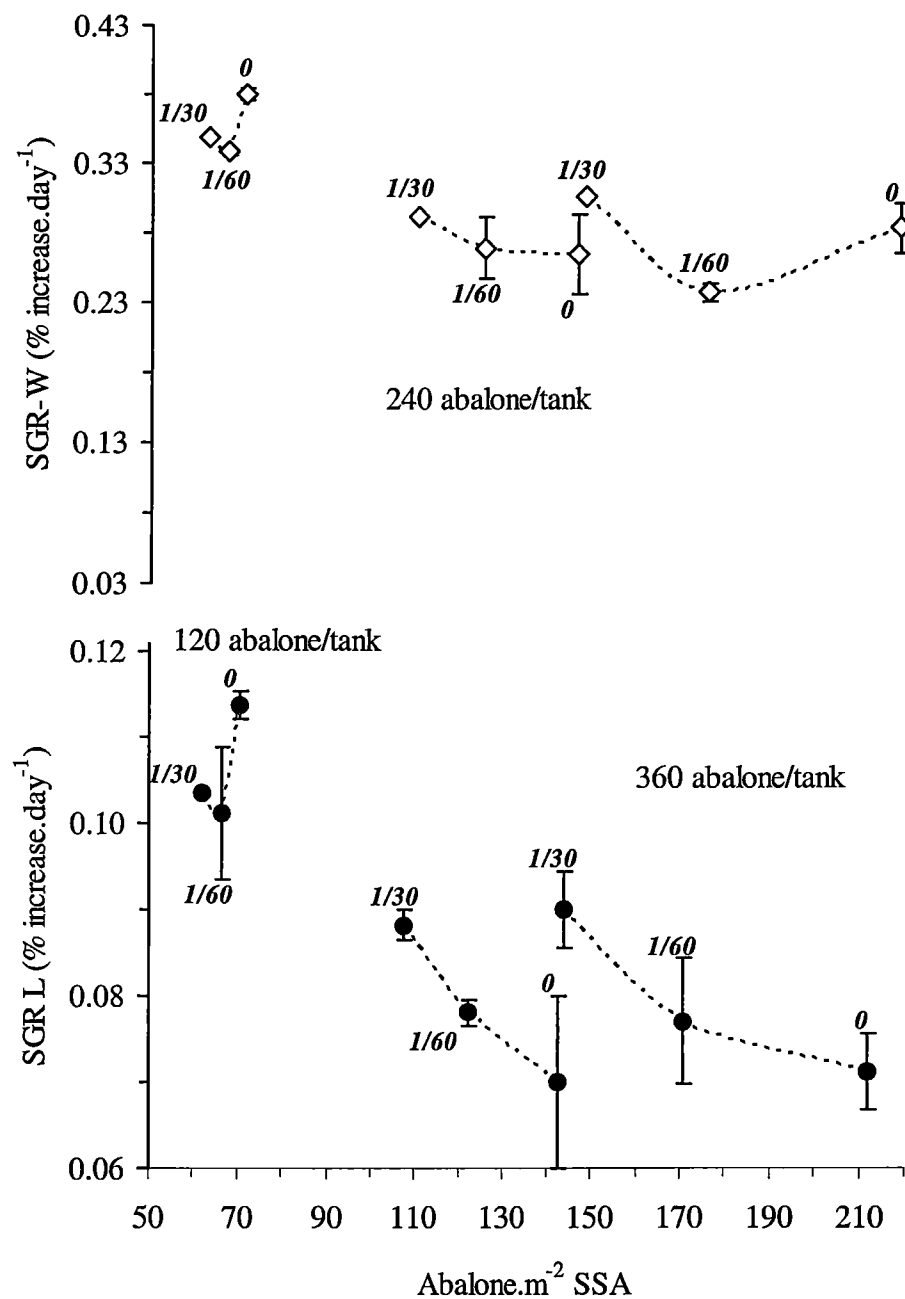


Figure 5.4 Growth data in terms of submerged surface area (SSA), which is defined as the submerged tank area plus one surface of the curved refuge area. Statistical analyses of the growth data are given in Figure 5.3, and Table 5.3. Numbers in italics indicate the level of refuge provision. Number of refuges per tank are given in Figure 5.1.

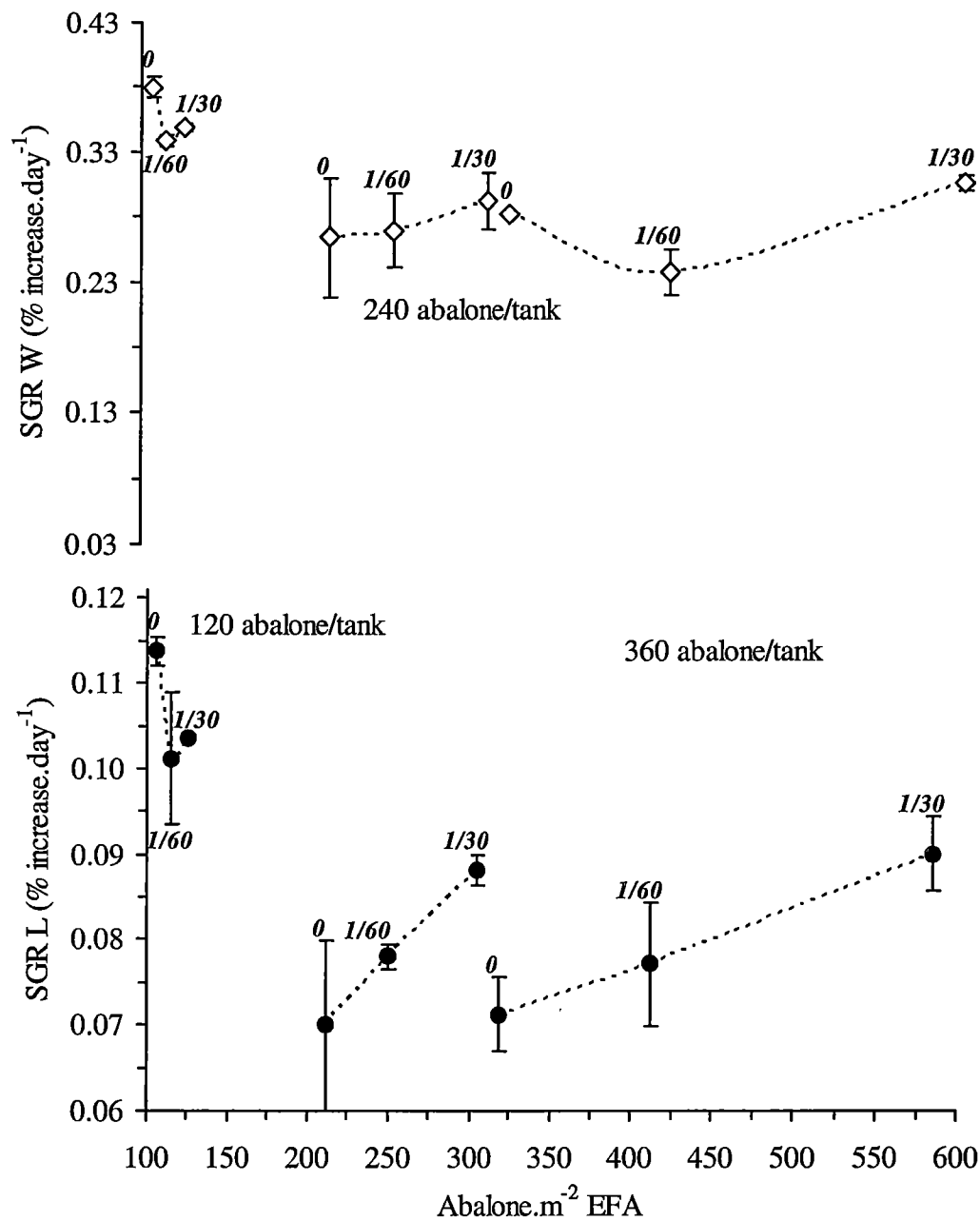


Figure 5.5 Growth data in terms of effective foraging area (EFA), which is defined as tank floor area minus the flat area covered by any refuges. Statistical analysis of the growth data are given in Figure 5.3 & Table 5.3. Numbers in italics indicate the level of refuge provision.

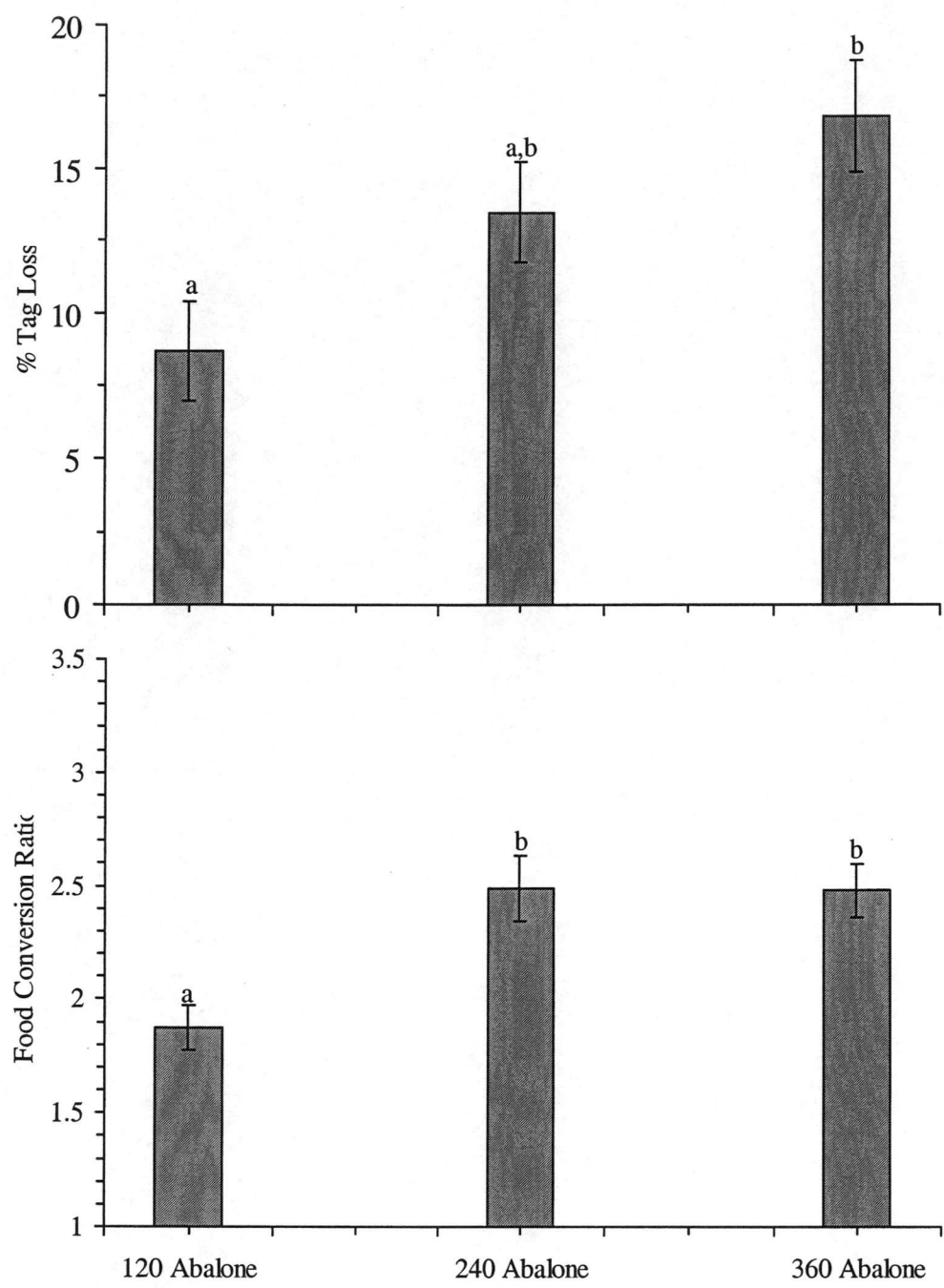


Figure 5.6 Tag Loss and FCR pooled for density (120, 240, 360 abalone per tank) ( $n=6 \pm \text{SE}$ ). Different superscripts indicate significantly different means based on ANOVA ( $P < 0.05$ ).

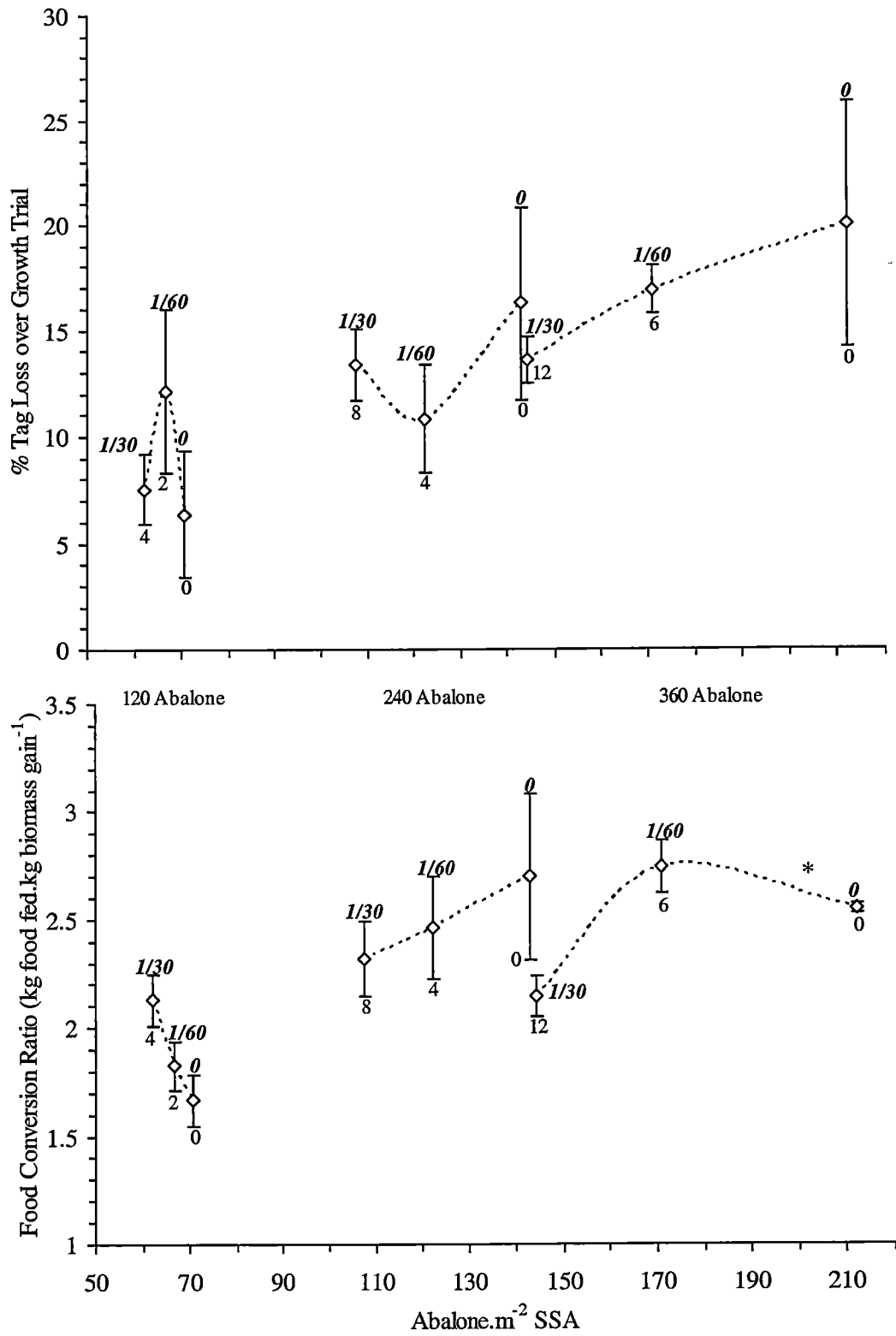


Figure 5.7 Tag loss and FCR in term of abalone per unit submerged surface area (SSA). Numbers above error bars (bold italics) indicate level of refuge provision; below error bars indicate number of refuges per tank. An asterisk indicates significant difference between no refuge (120/0, 240/0 & 360/0) and 1 refuge per 30 abalone (120/4, 240/8 & 360/12) based on individual t-tests ( $P < 0.1$ , Table 5.4).

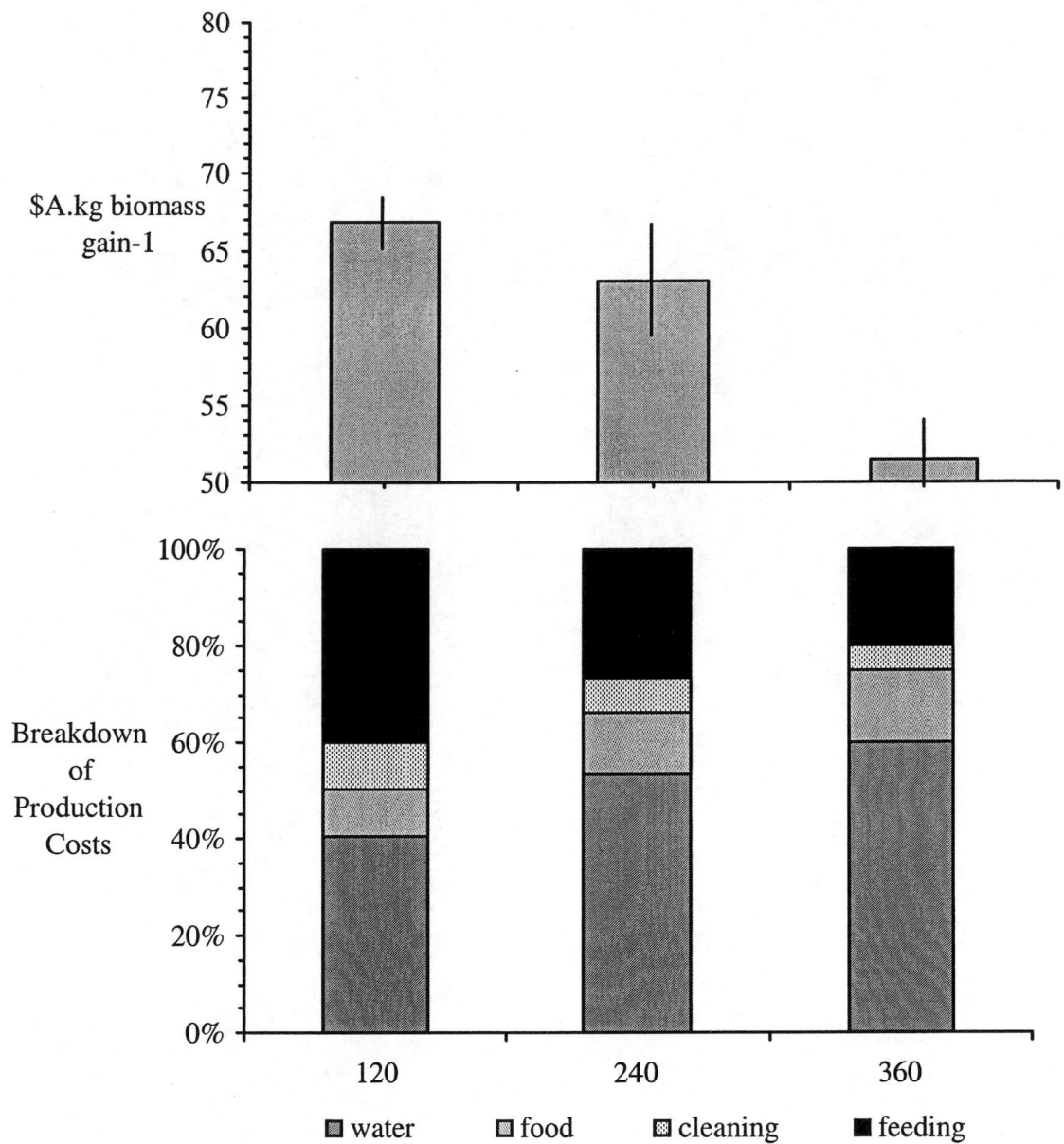


Figure 5.8 Cost of production as a function of abalone density (means $\pm$ SE, n=6). The model is based on the cost of water and food inputs, and labour for cleaning and feeding. Costs for water were based on 0.0001 c.L<sup>-1</sup> (S. Edwards, unpublished data) and food costs were taken as \$A2.70.kg (S. Edwards, unpublished data). Capital cost for tanks were included as an amortized cost over the trial (\$20/tank). Cost of labour for feeding and cleaning was taken as \$15.hr<sup>-1</sup>.



## CHAPTER 6

### Stocking Density And Refuge Provision In Relation To Abalone Behaviour

#### 6.1 Introduction

The culture of benthic, grazing gastropods such as abalone differs from pelagic species in the requirement for a solid attachment surface during both active foraging and inactive resting periods. In the wild, juvenile abalone of several species prefer a refuge during the inactive daylight resting period, from which they emerge to forage at night (Poore 1972; Shepherd 1973). During the active foraging period, abalone search for food items over the available submerged surface area (SSA). The use of negatively buoyant formulated diets in abalone culture restricts the availability of food items to the bottom surfaces of the tank, and the actual area where food items are accessible (ie. effective foraging area, EFA) may be further reduced by system design and the presence of refuges. However, this effectively increases the abundance of food items which has been shown to improve growth in tropical abalone (*H. assinina*) (Tahil & Menez 1999). Grazing gastropods generally have simple activity patterns, emerging to forage when the environmental cues for that species are suitable then grazing until various cues (either external, such as sunrise, or internal such as crop fullness) indicate that activity should cease and the quiescent phase should commence (Chelazzi *et al.* 1993; Della Santina *et al.* 1995). Some species return to the same spot that the foraging excursion originated from (ie. home) or to a different spot that is suitable for the quiescent period (Hartnoll & Wright 1977; Chelazzi *et al.* 1993; Della Santina *et al.* 1995; Gray & Hodgson 1997). Some limpets return on the same trail used for the outward excursion, while other species follow a random path on the outward and return journeys but still return to the same home

spot (Gray & Hodgson 1997). Studies of wild abalone stocks have also been shown to return to a home area within a reef, but not necessarily the same spot (Poore 1972; Shepherd 1973). Nakamura & Archdale (2001) report that in the Asian species of abalone they investigated, individuals which traveled less than 1m from the point of origin tended to return to that spot for the resting period. Assuming that refuges provided by the aquaculturist are suitable, provision of refuges for cultured abalone would be expected to make it easier to find either the home spot or a suitable resting area for the quiescent period, minimizing the expenditure of energy at the end of the foraging excursion. Provision of refuges therefore has the potential to substantially reduce energy expenditure in cultured abalone, by reducing the time required for foraging and reducing the effort required to find a suitable resting area. Locomotion is known to be energetically expensive compared to other organisms due to the requirement for mucus to lubricate the foot (Denny 1980; Donovan & Carefoot 1998).

Movement away from a local area in wild greenlip abalone populations has been shown to be related to available crevice area and food abundance, with very little movement of tagged abalone away from areas with an abundance of available crevice space (Shepherd 1973; Shepherd 1986a; Shepherd & Partington 1995). If density is increased in a given area, the available crevice area declines and abalone disperse to areas of lower density. Limiting available refuge area may therefore impose a stress on abalone through factors such as stacking (Douros 1987), where insufficient area for attachment to the substrate during the daytime quiescent period forces individuals to start attaching to the shells of conspecifics. The abalone species cultured in Australia are naturally aggregative and cryptic at the size class (<100mm) which is harvested for current markets (Shepherd 1973, 1986b), and there is a substantial body of literature on foraging behaviour and nocturnal activity in wild populations of herbivorous gastropods, and factors influencing this behaviour. An understanding of gastropod foraging behaviour and how this relates to the requirement for resting and foraging areas has obvious benefits for the aquaculturist. Incorporating this understanding into system design can optimise foraging activity, use of refuge areas and potentially improve growth. Combining data generated from the growth trial described in Chapter 5 with available literature on foraging behaviour on wild and cultured gastropods,

this chapter investigates abalone behaviour in relation to foraging activity and refuge provision, with the aim of further clarifying the mechanism through which stocking density and refuge provision influence growth.

## **6.2 Methods & Materials**

The system and experimental protocol is fully described in Chapter 5 (5.2.1 & 5.2.2). Briefly, the experimental system consisted of 18 circular fibreglass tanks with a central outlet, which were supplied with flow-through oceanic seawater. While influent water flow was set to  $1.8 \text{ L.min}^{-1}$  per 120 abalone, aquarium pumps were used to adjust the total internal water flow to  $12 \text{ L.min}^{-1}$  for each tank. Equal numbers of refuges perpendicular and parallel to the circular water current were supplied to treatments with refuges, which were held in position using poly-propylene rings filled with sand (Plates 6.5, 6.11, 6.18). Fresh food was provided every 2 days, and accumulated organic wastes removed every 4 days.

### ***6.2.1 Surface Area Calculations***

These calculations are fully described in Chapter 5 (5.2.3).

### ***6.2.2 Behavioural Observations***

Behavioural data are based on direct observation of the tanks, and photographs taken of the tanks through the trial both during the day and at night. Nocturnal observations and photographs were generally taken during summer, between 22.00-01.00, when the moon was not full.

At several points through the growth trial, the refuges were removed from the tanks, inverted and photographed. The tank was also photographed prior to the refuges being returned to the tank, with care being taken to return the refuges to the original position in the tank. Using these photos, the number of abalone on each refuge and on the tank floor under that refuge were counted, and combined to provide a total number of abalone using each refuge. These counts were averaged for individual replicate tanks, with these averages used in the statistical analysis of the data.

### ***6.2.3 Incidence of Shell Damage***

After being weighed at the end of the growth trial, 100 abalone from each tank were photographed to determine the incidence of shell damage. This was defined as chips or breaks visible in the growing shell edge from the photographs. The number of abalone from each tank with such damage was counted, then values for replicate tanks were pooled for statistical analysis.

### ***6.2.4 Statistical Analysis***

All analysed data was checked for heterogeneity of variance by observation of residual plots generated by JMP 3.2. No data required transformation. Unless otherwise specified, ANOVA was used to detect significant treatment effects using SPSS 10.02 (SPSS Inc.), and Tukeys HSD to determine differences between individual treatments. The data from the ANOVA (F, degrees of freedom, P) are shown in Table 6.2.

## **6.3 Results**

### ***6.3.1 Changes in Submerged Surface Area and Effective Foraging Area***

As SSA increased with the number of refuges at each density and abalone density decreased, effective foraging area (EFA) declined due to the use of half pipe sections as refuges (Figure 6.1). Any food items which landed on top of the refuges tended to slide down to the tank floor. At times some of the feed pellets were visible top of the refuges, but never more than 10% of the food available in the tank.

### ***6.3.2 Distribution***

Where refuges were provided, they were utilized by abalone during the day (Figure 6.2). Figure 6.2A shows that the average number of abalone per refuge was higher with 1 refuge per 60 abalone compared to 1 per 30 abalone for medium and high density treatments (44.033, 5, 6,  $P<0.001$ ). The total number of abalone under refuges was significantly higher at 1 per 30 abalone compared to 1 per 60 abalone for each density (402.35, 5, 6,  $P<0.001$ ) (Figure 6.2B). Doubling the number of hides per tank did not double the number of abalone utilizing them.

In terms of refuges parallel or perpendicular to the water current, significantly more abalone were under parallel refuges (188.07, 11, 12,  $P<0.001$ ), and there were significantly more abalone per parallel refuge, when 1 refuge was provided per 30 abalone (Figure 6.3) at 240 and 360 abalone per tank (18.088, 11, 12,  $P<0.001$ ). There was no effect of refuge orientation at 120 abalone per tank, or with 1 refuge per 60 abalone at any density.

### ***6.3.3 Behavioral Observations***

It was evident from observing the tanks that when available, the majority of abalone preferred to spend the quiescent day-time period under a refuge (eg. compare Plates 6.13, 6.15 & 6.17). In tanks with no refuges, the abalone preferred to spend this period around the outside edge of the tank at the junction of wall and floor (Plate 6.1). As density increased, abalone tended to spread more into the middle of the tank rather than up the walls (compare Plate 6.1 with Plates 6.7, 6.13). While complete stacking was uncommon during the day (one abalone completely covering a second), partial stacking was more evident as density increased (Plates 6.7, 6.13).

Nocturnal observations showed that abalone would move through lighter accumulations of organic wastes, but tended to avoid more heavily fouled areas of the tank (Plates 6.8, 6.10 & 6.12). This was also evident from disturbance patterns in the organic wastes observed during the day (Plates 6.5, 6.13).

Abalone were also observed moving over the heavier accumulations of wastes on several occasions.

Abalone were commonly observed crawling over all the available submerged surface area, such as the tops of the refuges and the polypropylene rings securing the refuges (Plate 6.10). While actively foraging abalone that came in contact with other foraging abalone tended to change direction rather than continue in the same path and crawl over each other, this was at times observed (Plates 6.2, 6.8, 6.10). A number of abalone in each tank were also observed to be “alert” (shell elevated off the substrate, feelers extended), but not actively foraging.

At any one time only 60-80% of the total abalone in any given tank appeared to be active (shell elevated off the substrate and feelers extended), although the actual number observed to be active was not counted.

#### **6.3.4 Water Currents**

Observation of water currents using cochineal food dye revealed that all treatments had some degree of water movement around the outer wall of the tank for the full depth of the water column. Observations of dye movement both in situ and from video footage indicated that the water current at the outer wall in tanks with either 12 refuges (360 abalone/tank) or 8 refuges (240 abalone/tank) was approximately halved compared to the same density with no refuges. As the number of refuges per tank increased with the level of refuge provision at medium and high densities, the water movement in the central area of the tank decreased and was virtually non-existent at 12 refuges per tank.

Water movement through the refuges was evident in all treatments, but decreased as the number of refuges per tank increased. Being in line with the water flow, water exchange was better under refuges parallel to the circular water current than those perpendicular to it. While water movement through refuges parallel to the water flow was a result of being in line with the water current, water exchange through the perpendicular refuges appeared to be generated by a weak venturi action drawing water through the refuge from the central area of the tank.

#### **6.3.5 Shell Damage**

Although damage to the growing shell edge was observed in all density treatments, ANOVA showed that the prevalence of damage to this growing edge did not differ between treatments, based on photographic analysis (data not shown). However, visual observations indicated that the growing edge tended to be thinner in abalone held at the lowest density.

#### **6.4 Discussion**

During the day period abalone are generally inactive, with cryptic species preferring some form of refuge. As density increases, competition increases for primary attachment space and abalone will start attaching to each other during the quiescent period (ie stacking). Observations from photographic records showed that in treatments with no refuges, the incidence of stacking increased with density (compare Plates 6.1, 6.7, 6.13). Douros (1987) also found the incidence

of stacking was related to density in wild abalone, and it has been suggested that stacking in cultured abalone restricts foraging behaviour and therefore contributes to decreased growth through food limitation and con-specific interactions (Mgaya & Mercer 1995; Capinpin *et al.* 1999). Such effects would explain why growth rates were lower in the 240/0 & 360/0 treatments compared to 120/0 (Chapter 5). The increase in crowding at medium and high densities would lead to an increased interaction between individuals, which would also explain the overall increase in tag loss with density (Chapter 5, Figure 5.6). While increasing the level of refuge provision at constant density increased the surface area available for primary attachment (Figure 6.1), the % cover of the SSA declined (Figure 5.1) and the incidence of stacking outside the refuges decreased (compare Plate 6.7 with Plate 6.9 & 6.11, Plate 6.13 with Plates 6.15 & 6.17). There was a degree of stacking under refuges at 1 refuge per 60 abalone, which was reduced when the level of refuge provision was increased to 1 refuge per 30 abalone (compare Plates 6.21 & 6.22, 6.23 & 6.24), as the abalone were able to disperse over a greater surface area. This may explain why the level of tag loss declined as more refuges were provided at medium and high densities (Chapter 5, Figure 5.7). At 120 abalone per tank, density was probably low enough not to have been an issue even with no refuges.

The presence of refuges allows the foraging and resting areas to be separated, preventing inactive abalone being disturbed by those actively foraging (Plates 6.8 & 6.12, 6.14 & 6.18). Studies with different species of abalone have shown that active foraging does not occur on every night (Uki 1981; Donovan & Carefoot 1998; Knauer *et al.* 1995; Nakamura & Archdale 2001), which has also been reported in wild populations of herbivorous gastropods (Hartnoll & Wright 1977; Little & Stirling 1985; Evans & Williams 1991; Gray & Hodgson 1997). Five different levels of activity between fully quiescent and fully active have been described in abalone (Donovan & Carefoot 1998). Ingested ration passes through the abalone crop in 18-24 hours (Britz *et al.* 1996), and if crop fullness was the only cue involved in initiating a foraging excursion then all the abalone would be active on every night. The fact that this does not occur indicates that there are factors which control active foraging other than just gut fullness (Chelazzi *et al.* 1993), and increasing the available surface area for resting may be important in

allowing the abalone to remain quiescent without being disturbed by active abalone. Increasing the SSA by increasing the level of refuge provision reduced the degree of crowding under the refuges (compare Plates 6.21 with 6.22, 6.23 with 6.24), which would make it easier for foraging abalone to successfully emerge from the refuge.

Food density will effectively increase as the level of refuge provision at each density, due to the concomitant decrease in EFA (Figure 6.1, see also Figure 5.1). It should also be noted that, as Figure 6.1 shows, foraging abalone will further reduce EFA as they represent a surface area where no food will be available. It would be expected that an increase in food abundance will minimize the duration of foraging excursions, assuming gut fullness is one of the cues controlling the length of a particular foraging excursion (Chelazzi *et al.* 1993; Della Santina *et al.* 1995). Once the crop is full, there is no longer any need to expend energy on locomotion and risk predation by further foraging activity, so the urge to forage is replaced by the urge to return to a suitable resting area. Increasing the level of refuge provision would reduce the effort required to locate a suitable resting area for the quiescent period, especially if there is some form of homing activity involved. A number of herbivorous gastropods show varying degrees of homing behaviour, ranging from returning to exactly the same spot on a rock to resting in the same general area after each foraging excursion, with some species showing different levels of homing behaviour in different populations (Della Santina *et al.* 1995). Some species return on the same trail used for the outward excursion, following a chemical cue such as a mucous trail, while others follow a random path for both the outward and return components of the excursion, utilizing some other cue to navigate back to the home spot (Gray & Hodgson 1996). The importance of homing in abalone has not been addressed, although both wild and cultured abalone have shown some degree homing behaviour (Shepherd 1973; Nakamura & Archdale 2001).

It is clear from the results presented here that greenlip abalone prefer a refuge during the quiescent resting period, with observations showing that as refuges were provided, abalone tended to use them (compare Plates 6.7 with 6.9 & 6.11, Plates 6.13 with 6.15 & 6.17). This is confirmed by Figure 6.2, which shows the total number of abalone under refuges increasing as they became available. At



medium and high densities, the refuges were relatively crowded at 1 refuge per 60 abalone, compared with 1 refuge per 30 abalone (compare Plates 6.19 & 6.20, 6.21 & 6.22, 6.23 & 6.24). As discussed above, this may have contributed to the improvement in growth rates as the level of refuge provision increased.

From the discussion above, it can be seen that provision of refuges and increasing the level of refuge provision at a constant density has the potential to reduce the energy required for locomotion by making it easier to commence a foraging excursion, shorten the duration of these excursions and facilitate the location of a suitable resting area for the quiescent period. The energy requirement for gastropod locomotion and its mucous component is well established (Denny 1980; Peck *et al.* 1987; Donovan & Carefoot 1988), and any reduction in locomotive activity allows more energy to be available for somatic growth. This could explain the increase in growth rates observed as the level of refuge provision increased from 0 to 1 per 30 abalone at medium and high densities (Chapter 5, Figure 5.4 & 5.5).

The submersible aquarium pumps were used to standardise the internal water flow between treatments (Chapter 5, Table 5.1). Observations with a food dye showed that while the pumps did maintain a circular water flow around the outside of the tank in all treatments, provision of refuges did alter the hydrodynamics of water movement within the tank. Circular water currents were evident in all treatments, although the velocity declined as the number of refuges per tank increased. Refuges parallel to the water current tended to have better water exchange than those perpendicular to it, which may explain the preference shown in Figure 6.3 for refuges parallel to the water current. This effect was evident only at the higher level of refuge provision (one refuge per 30 abalone) at medium and high densities, which suggests that in these treatments there was sufficient refuge area for abalone to select a preferred resting spot. At the lower level of refuge provision (1 per 60 abalone), the restricted refuge area and associated crowding prevented this preference being expressed.

Water currents through the perpendicular refuges were generated by a weak venturi action drawing water from the central area of the tank, while water currents in parallel refuges was a consequence of being in line with the water flow. Figure 6.3 shows that as competition increased for refuge area at medium

and high densities, there was a preference for refuges parallel to the water current. Haliotids are known to orient themselves to optimise water flow past the respiratory openings in the shell (Voltzow 1983; Tissot 1992), and it is conceivable that there is a preference for the refuge which will provide conditions favouring this optimal flow.

This trial cannot determine whether the abalone preferred the refuges as refuge from light or as a surface to rest on during the period of inactivity. Although the tanks were covered with black plastic, some light may have leaked through small holes in the covers, and the covers were also removed for cleaning, feeding and daily observations. If the observed results were purely an effect of increased SSA, then it is likely that abalone would have chosen the outside of the refuges as well as the inside. Plates 6.5, 6.9 & 6.17 show that there was a definite preference for being under the refuge rather than on the outside the refuges. This would tend to suggest that there is a selective pressure operating for a refuge rather than just primary attachment area.

It is evident from the results presented here and in Chapter 5 that density and provision of refuges will influence abalone behaviour and bulk water quality. Chapters 7 & 8 will investigate the effect of accumulated organic wastes and abalone metabolism on water quality.

### **6.5 Conclusions**

As the level of refuge provision at a given density increases and more refuges become available, more abalone use them although the relationship is not linear for the system used in this trial. This reduced crowding during the resting period would have made it easier for individual abalone to emerge and begin foraging. As only a percentage of abalone are active on any one night, this would also have minimized any disturbance of inactive abalone. The decrease in effective foraging area with the increase in refuges per tank also effectively increased food density, which would have minimized the energy expenditure on foraging. It is also likely that provision of refuges would decrease the energy required to locate a suitable spot for the quiescent daytime period.

A preference for refuges parallel to the water current in the tanks was evident when 1 refuge was provided per 30 abalone, but not at 1 refuge per 60 abalone. This was probably linked to better water exchange under these refuges.

Treatments	Submerged Surface Area (m <sup>2</sup> )*	Abalone.m <sup>-2</sup> SSA	% Cover of SSA	Effective Foraging Area (m <sup>2</sup> )**	Abalone. m <sup>-2</sup> EFA
120/0	1.70	71	15	1.13	106
120/2	1.8	67	14	1.04	115
120/4	1.93	62	13	0.96	125
240/0	1.68	143	29	1.13	212
240/4	1.96	122	25	0.96	251
240/8	2.23	108	22	0.77	305
360/0	1.70	212	43	1.13	319
360/6	2.11	171	35	0.87	413
360/12	2.50	144	30	0.61	586

Table 6.1 Surface areas from experimental treatments. Submerged surface area was calculated as the submerged surface area of the tank (walls+floor+one side of the refuge). In calculating the % cover, it was determined that each abalone covered 80% of a circle with a diameter equal to its length, as described in Chapter 5 (5.2.3). Effective foraging area was calculated as the floor area of the tank minus the flat area covered by the refuges. These calculations are described in full in Chapter 5 (5.2.3).

Variable	n	F ratio	Degrees of Freedom	P
<i>Abalone Under Refuges</i>				
Total Under	2	402.352	5, 6	0.000
Average per Refuge	2	44.033	5, 6	0.000
Paralell vs Perpendicular (Total Under)	2	188.072	11, 12	0.000
Paralell vs Perpendicular (Average per Refuge)	2	18.088	11, 12	0.000
Tag Loss	6	5.150	8, 15	0.020

Table 6.2 Data from ANOVA performed on the variables indicated. Tukeys HSD was used as a post-hoc test to compare individual means, and observation of residual plots to asses homogeneity of variance. Distribution data was analysed in terms of individual treatments (n=2), and tag loss was pooled for density (n=6).

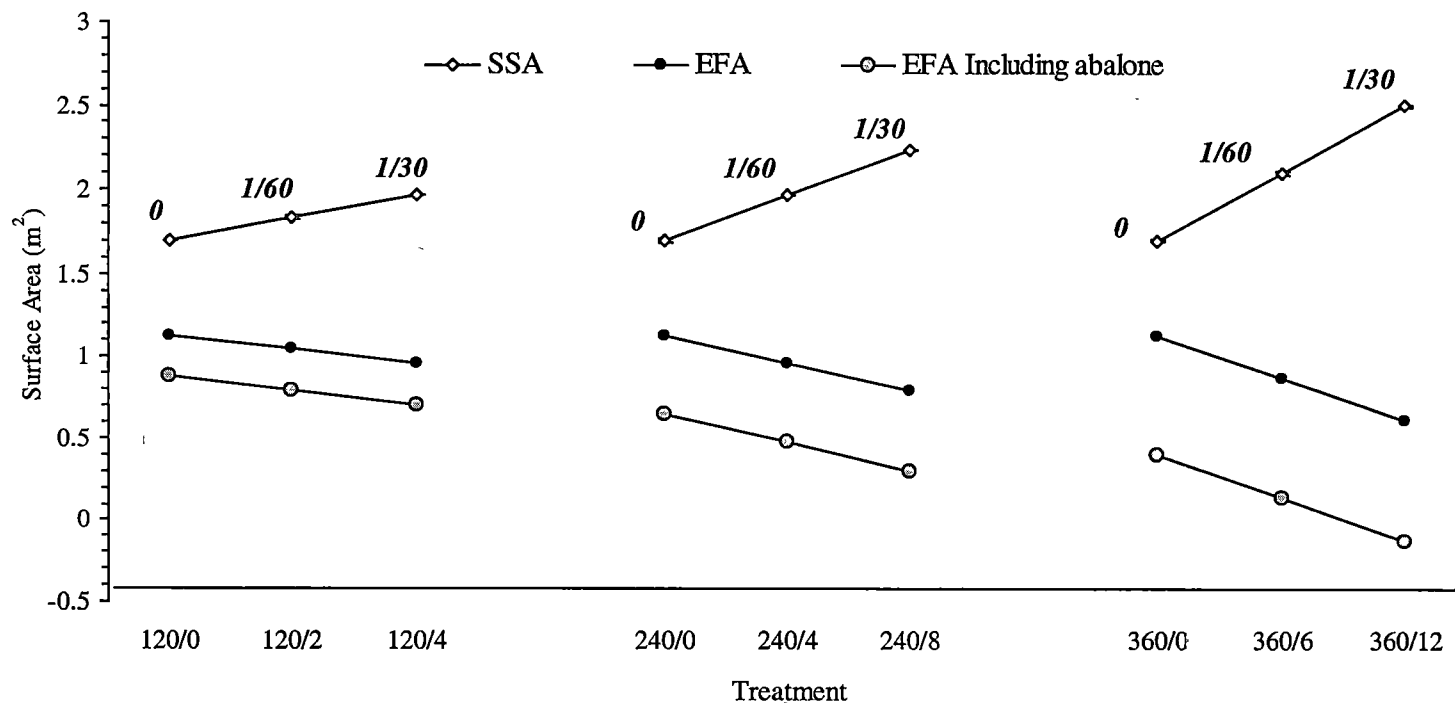


Figure 6.1 Submerged surface area (SSA) and effective foraging area (EFA) for experimental treatments. SSA is defined as the submerged area of the tank available for the daytime resting period (walls, tank floor and one curved surface of refuge). EFA is defined as the area where food is available for grazing (floor area of the tank minus the flat area covered by the refuges). Numbers below the axis are abalone per tank/number of refuges per tank. Numbers in italics indicates the level of refuge provision.

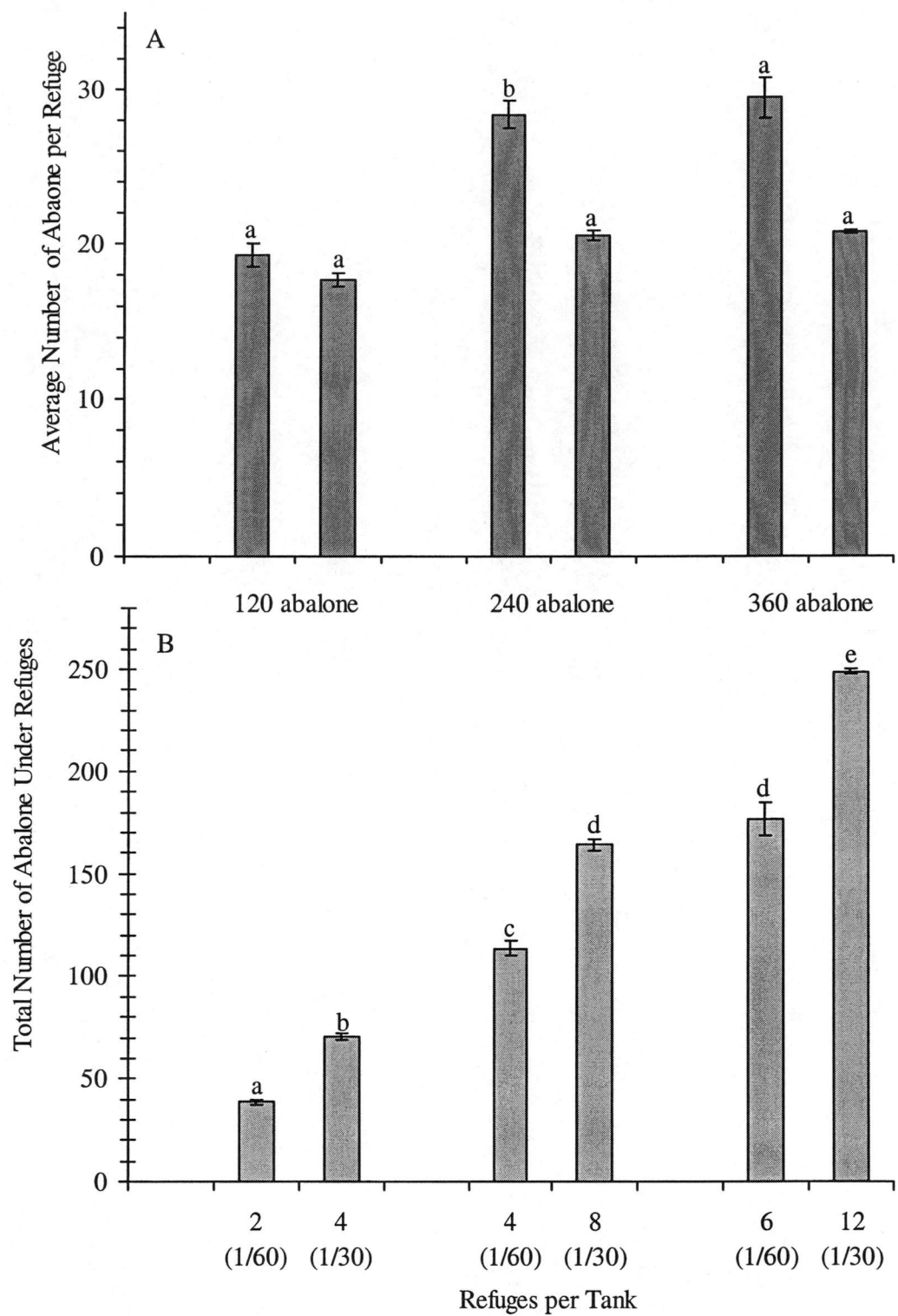


Figure 6.2 Numbers of abalone per refuge and under refuges during the day, determined by photographic analysis (means $\pm$ SE,  $n=2$ ). Different superscripts indicate significantly different means ( $P < 0.05$ ). Numbers in brackets indicate level of refuge provision (1 refuge per 30 or 60 abalone)

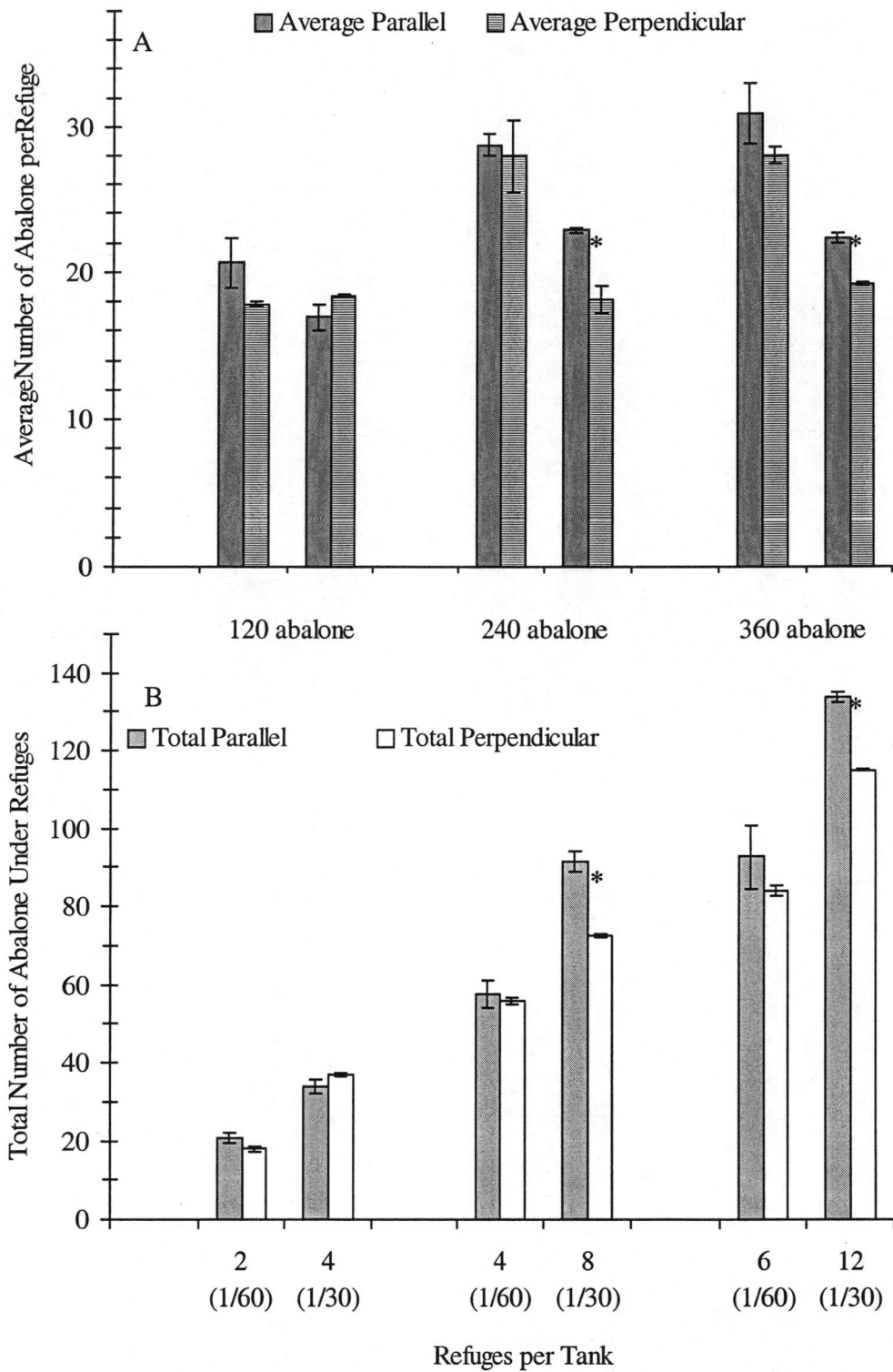


Figure 6.3 Distribution between refuges parallel and perpendicular to the circular water flow (means $\pm$ SE, n=2). Each tank had equal numbers of refuges in each orientation and refuges per tank is the total number of refuges per tank. Asterix indicates a significant difference between parallel and perpendicular refuges ( $P < 0.05$ ). Numbers in brackets indicate level of refuge provision (1 per 30 or 60 abalone).



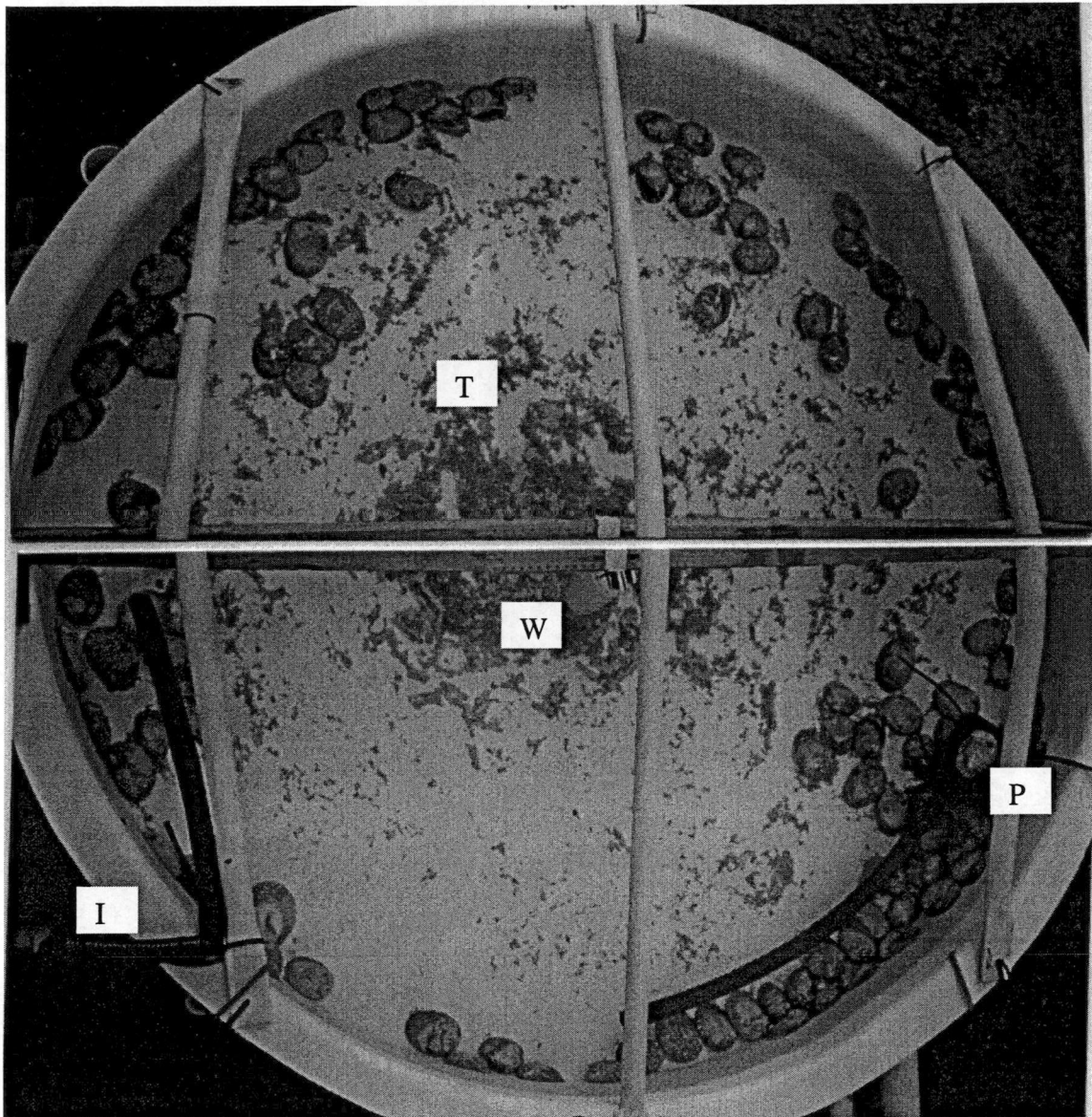


Plate 6.1 Treatment 1 during the day (120 abalone, no refuges), just prior to being cleaned.

Notice the accumulated organic wastes, and the tracks that abalone have made through the lighter accumulations. Heavier accumulations are not disturbed.

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= 30 cm

W = accumulated organic wastes

I = Water inlet

P = Submersible aquarium pump

T = Tracks pushed through organic wastes by abalone

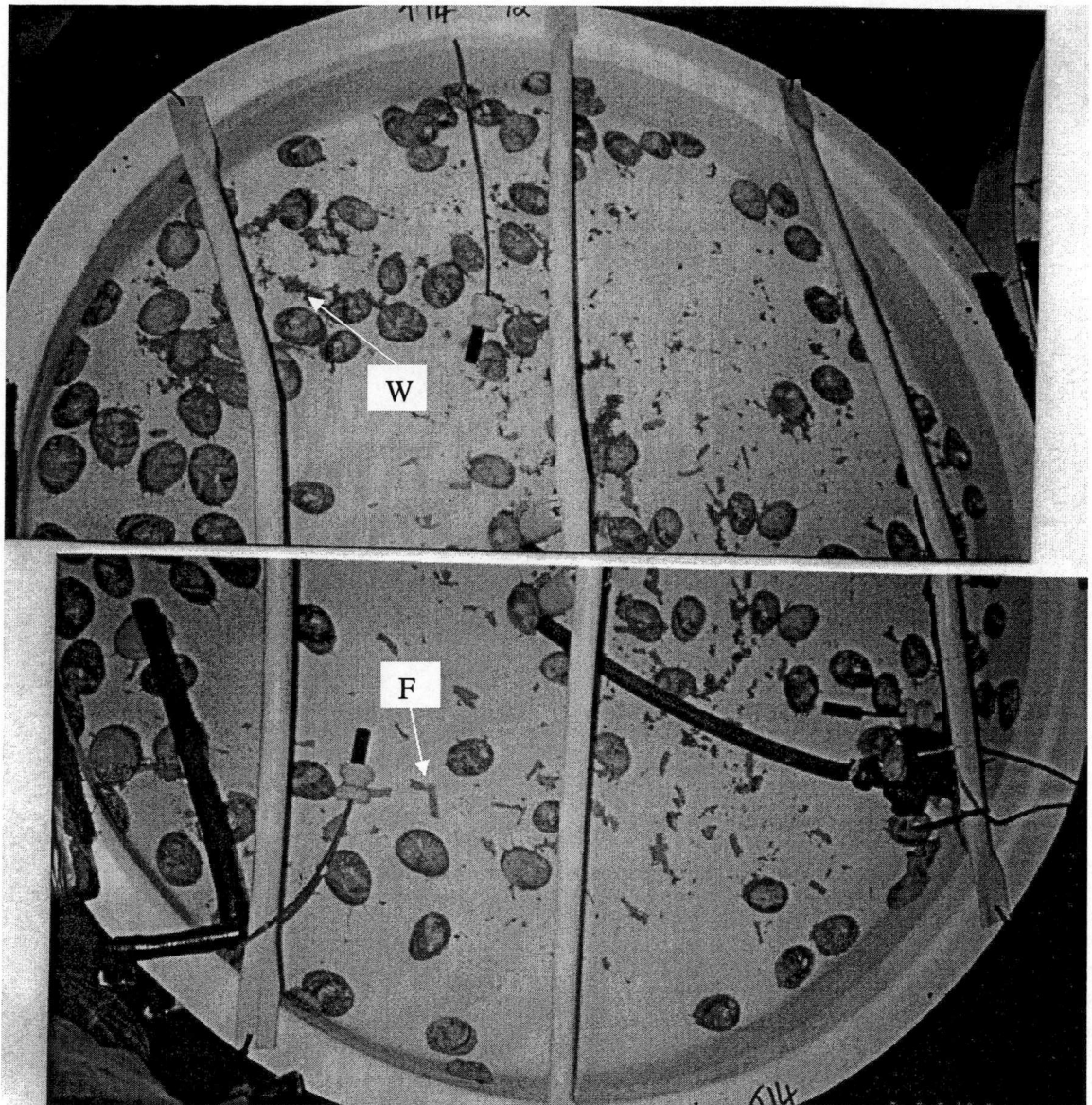


Plate 6.2 Treatment 4 at night (120 abalone, no refuges). The tanks had been cleaned during the day, and abalone can be seen actively foraging. Organic wastes, mainly faeces at this stage, are already starting to accumulate. Fresh ration is visible on the tank bottom. Foraging abalone have dislodged the submersible pump, which happened infrequently.

————— = 30 cm

W = accumulating organic wastes

F = fresh ration

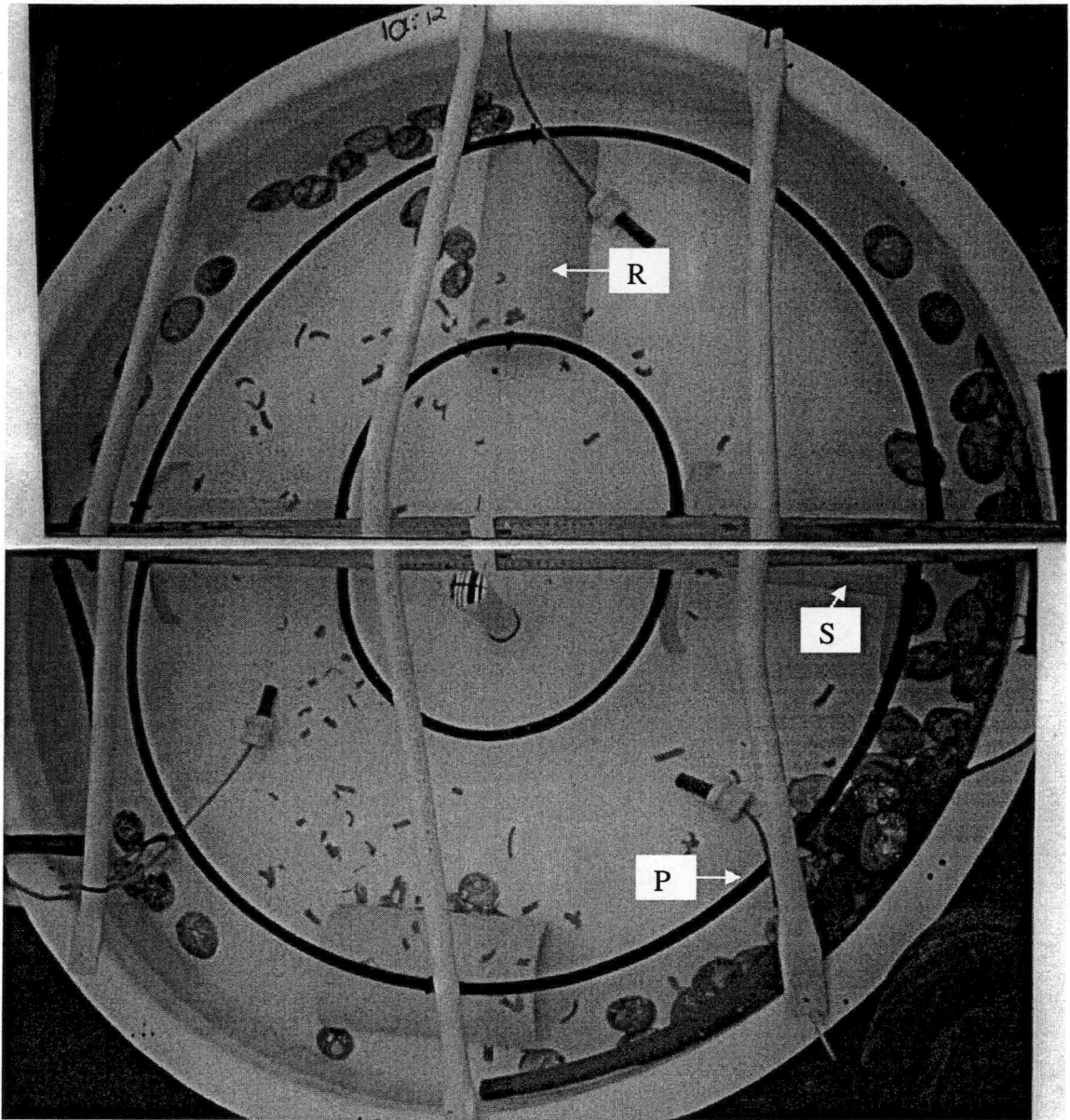


Plate 6.3 Treatment 2 during the day (120 abalone, 2 refuges). The tanks had just been cleaned, with the daily ration clearly visible. Abalone not under refuges are gathered around the outer edges of the tank

————— = 30 cm

P = weighted poly-propylene rings holding refuges in position  
R = refuge, secured to the polypropylene ring with cable ties.  
S = support for polypropylene in the absence of refuges



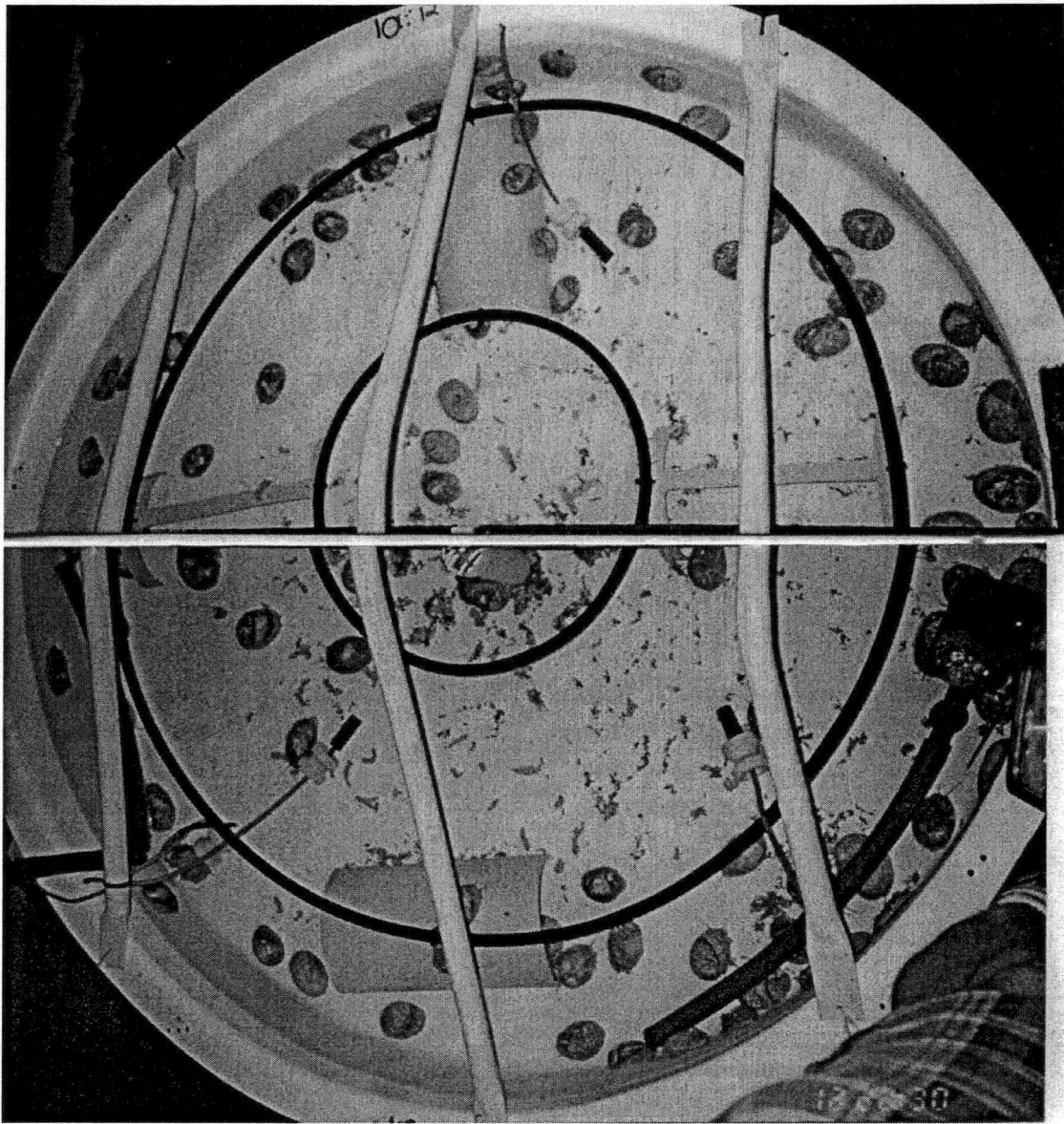


Plate 6.4 Treatment 2 at night (120 abalone, 2 refuges).

———— = 30 cm

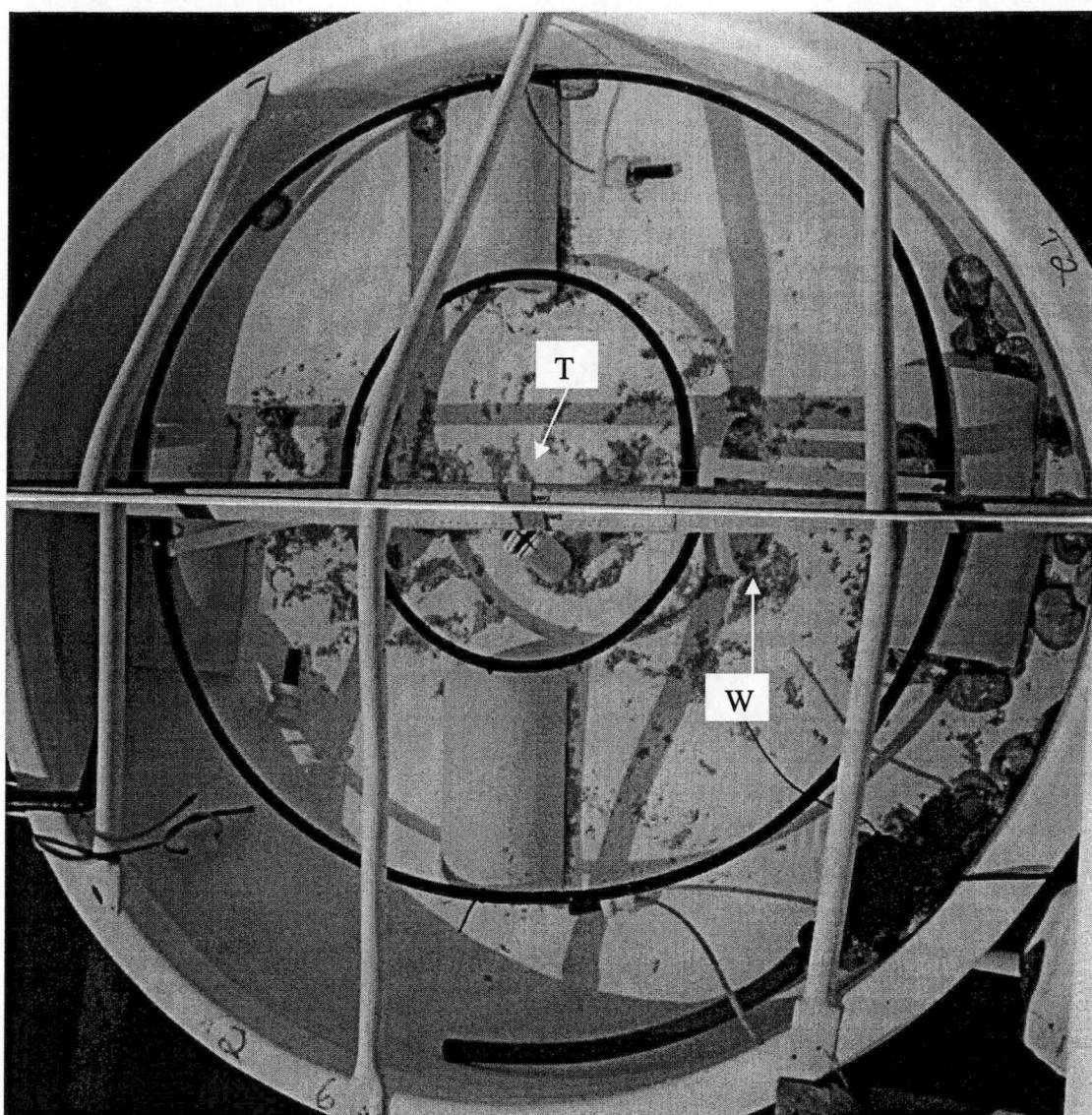


Plate 6.5 Treatment 3 during the day (120 abalone, 4 refuges). Fewer abalone are visible outside the refuges than in Plate 6.3. Notice also the tracks pushed through the accumulated organic wastes. This tank had been cleaned the previous day, and was due to be cleaned the following day.

————— = 30 cm.

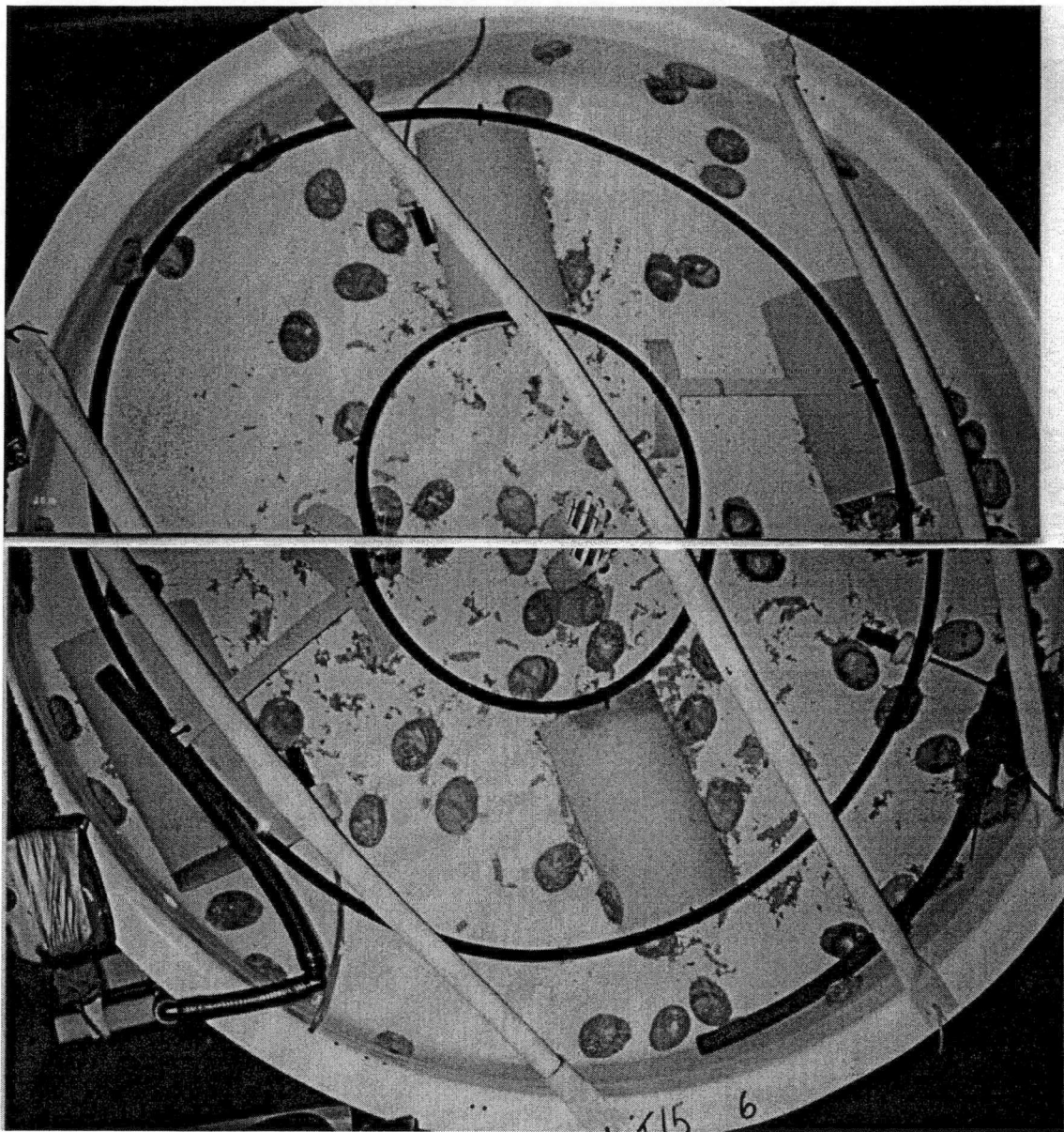


Plate 6.6 Treatment 3 at night (120 abalone, 4 refuges). This tank had been cleaned during the day, and fresh ration is visible on the floor of the tank.

————— = 30 cm



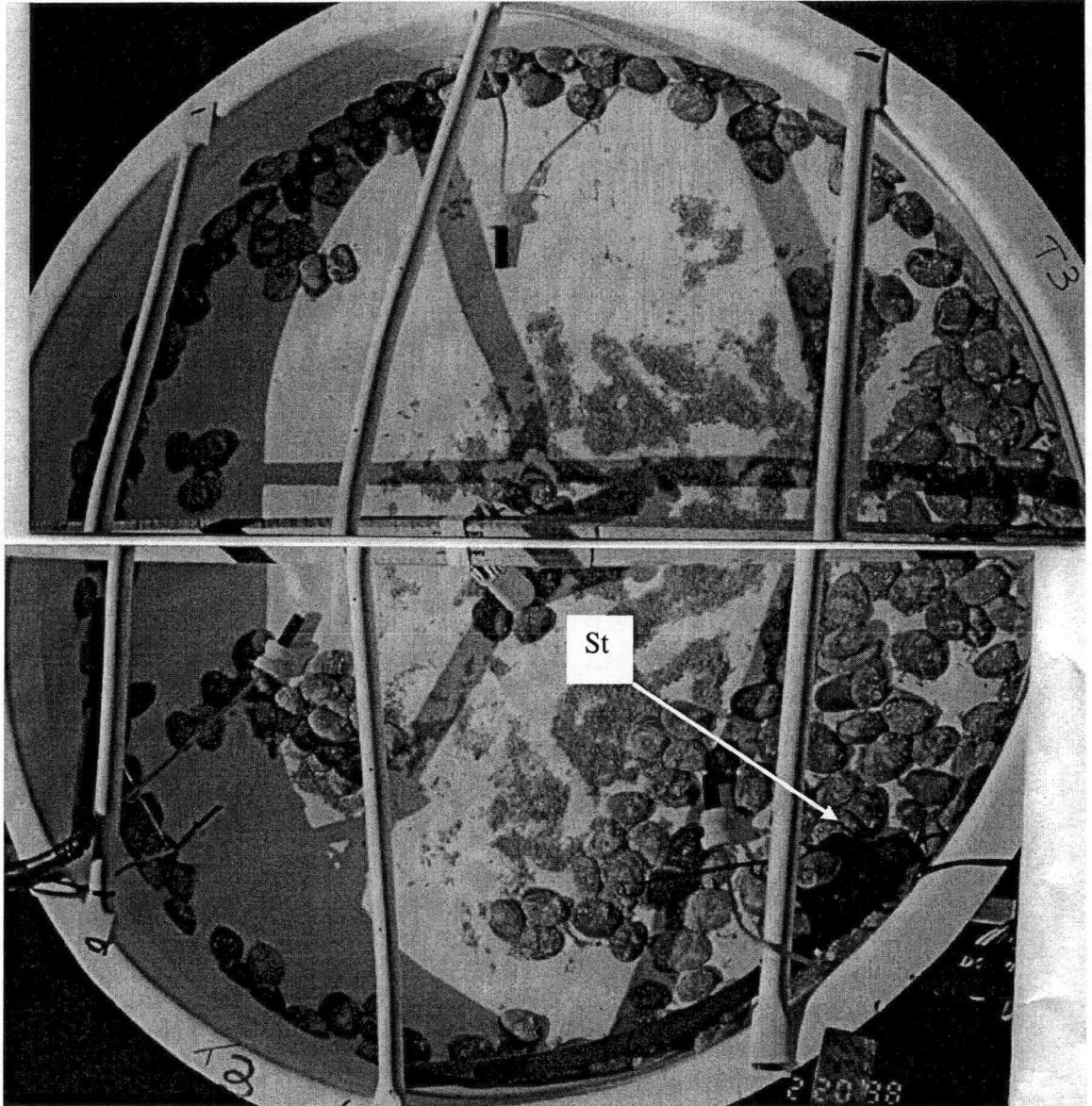


Plate 6.7 Treatment 4 during the day (240 abalone, no refuges). Abalone tend to gather around the edges, and tracks are visible through the wastes. A degree of partial stacking is also evident

————— = 30 cm

St – stacked abalone (one abalone attached to the shell of a second)

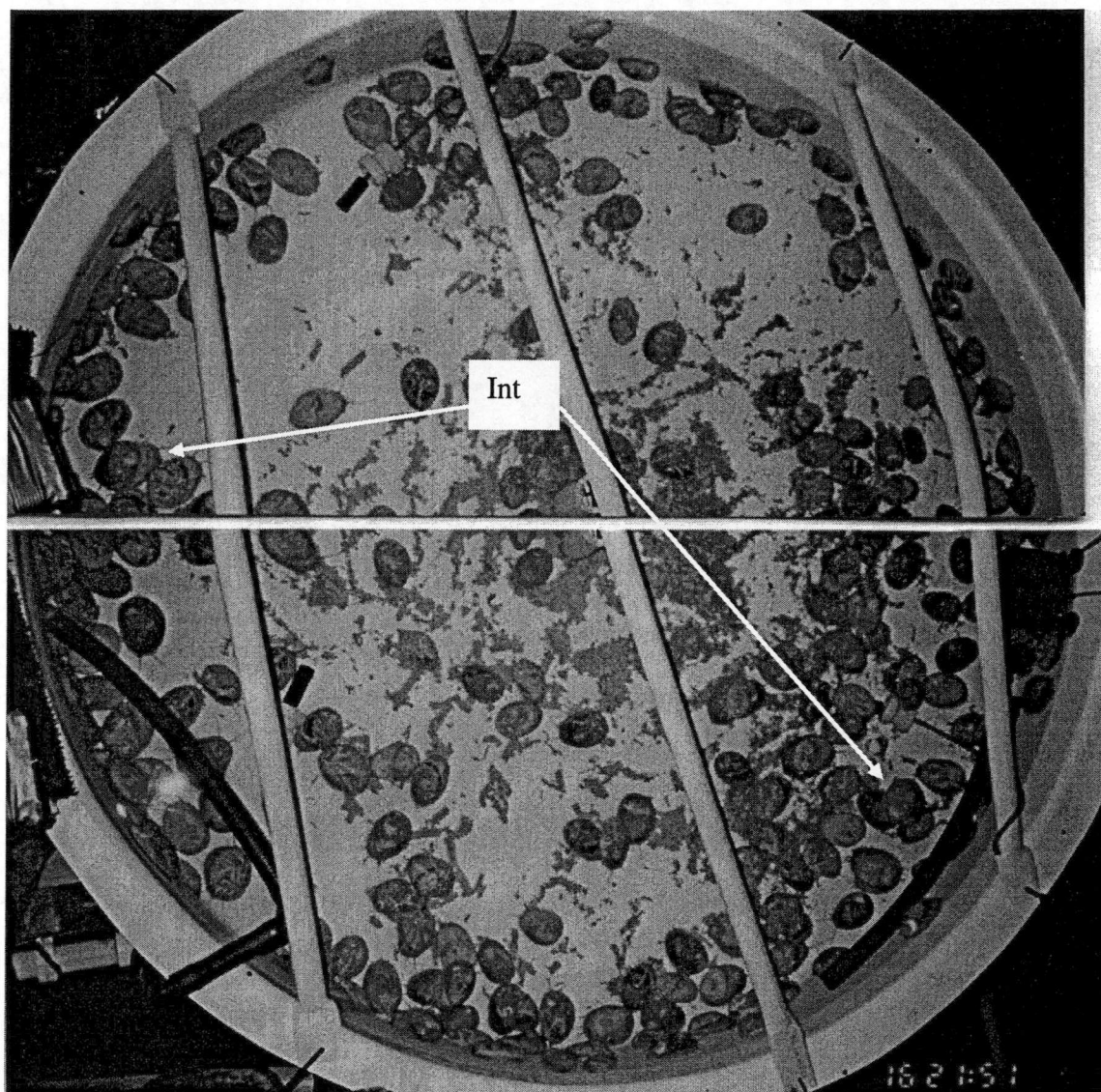


Plate 6.8 Treatment 4 at night. Abalone can be seen pushing through the light accumulations of organic wastes, but not the more heavily fouled areas. Interactions between foraging abalone can also be seen (one abalone running into another).

———— = 30 cm

Int – Interactions between foraging abalone.



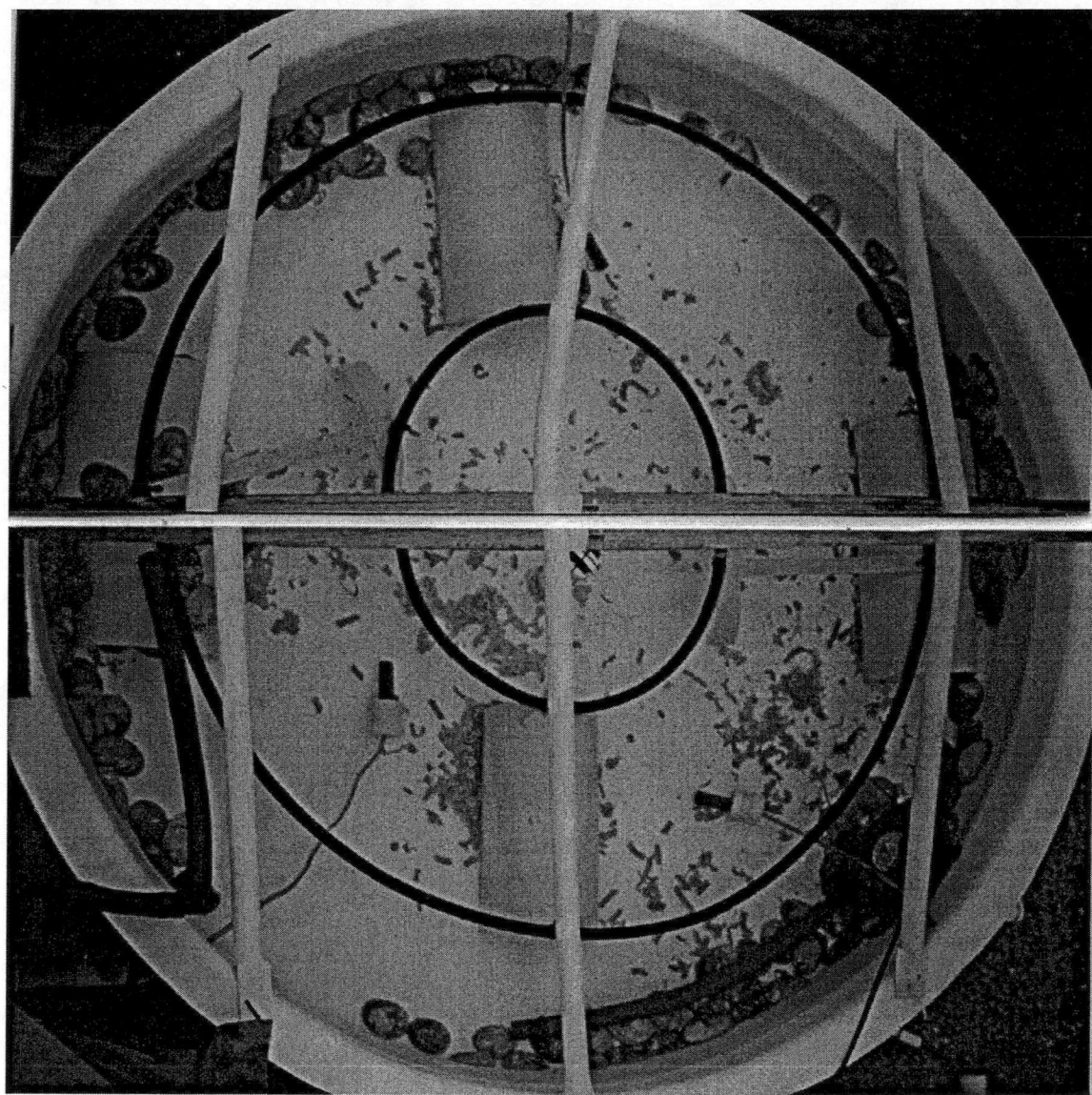


Plate 6.9 Treatment 5 during the day (240 abalone, 4 refuges). Abalone under refuges are gathered around the edge of the tank, or along the edge of the refuges.

————— = 30 cm

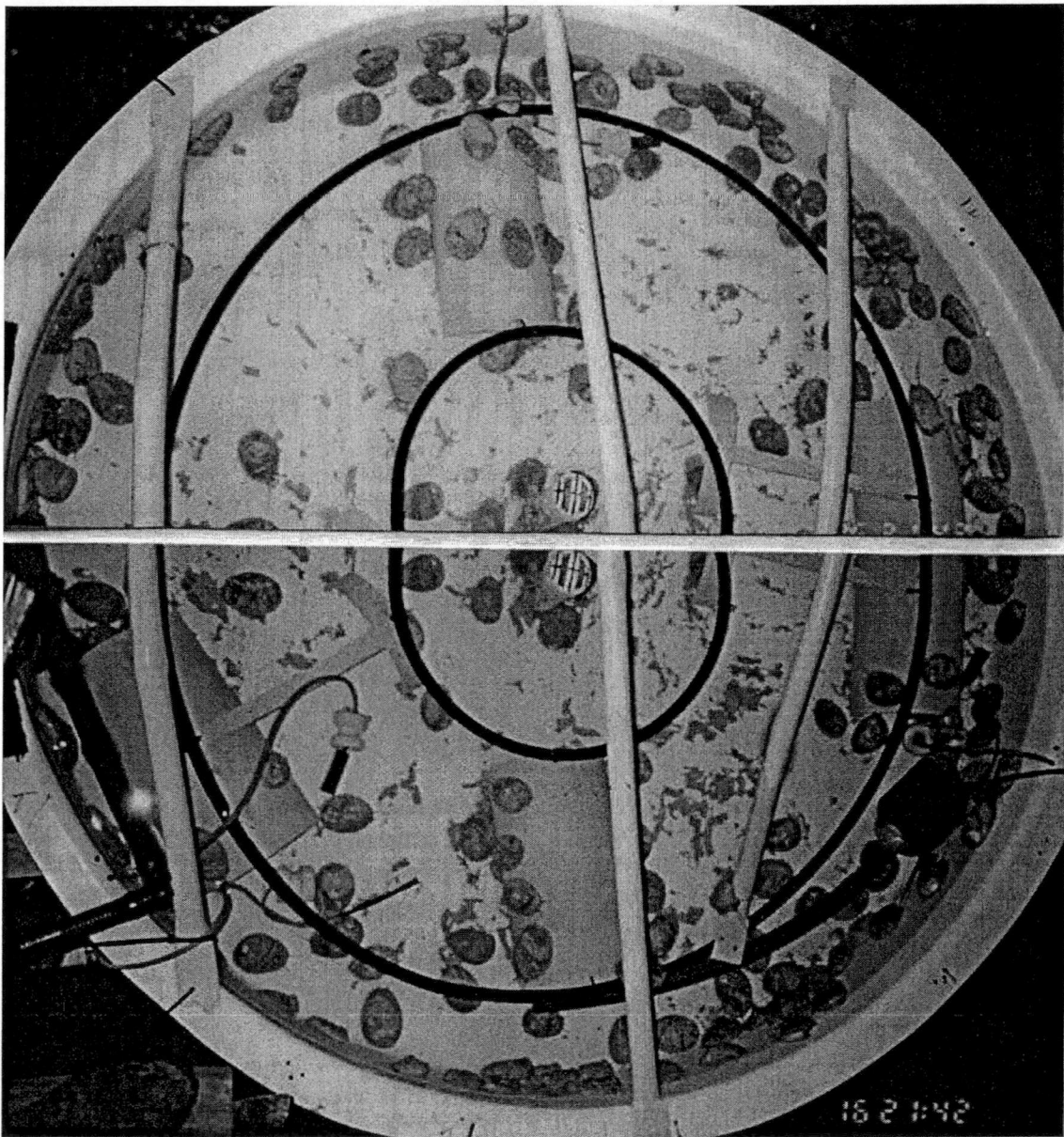


Plate 6.10 Treatment 5 at night. Abalone are moving over all the available surfaces, including the tops of the refuges.

————— = 30 cm

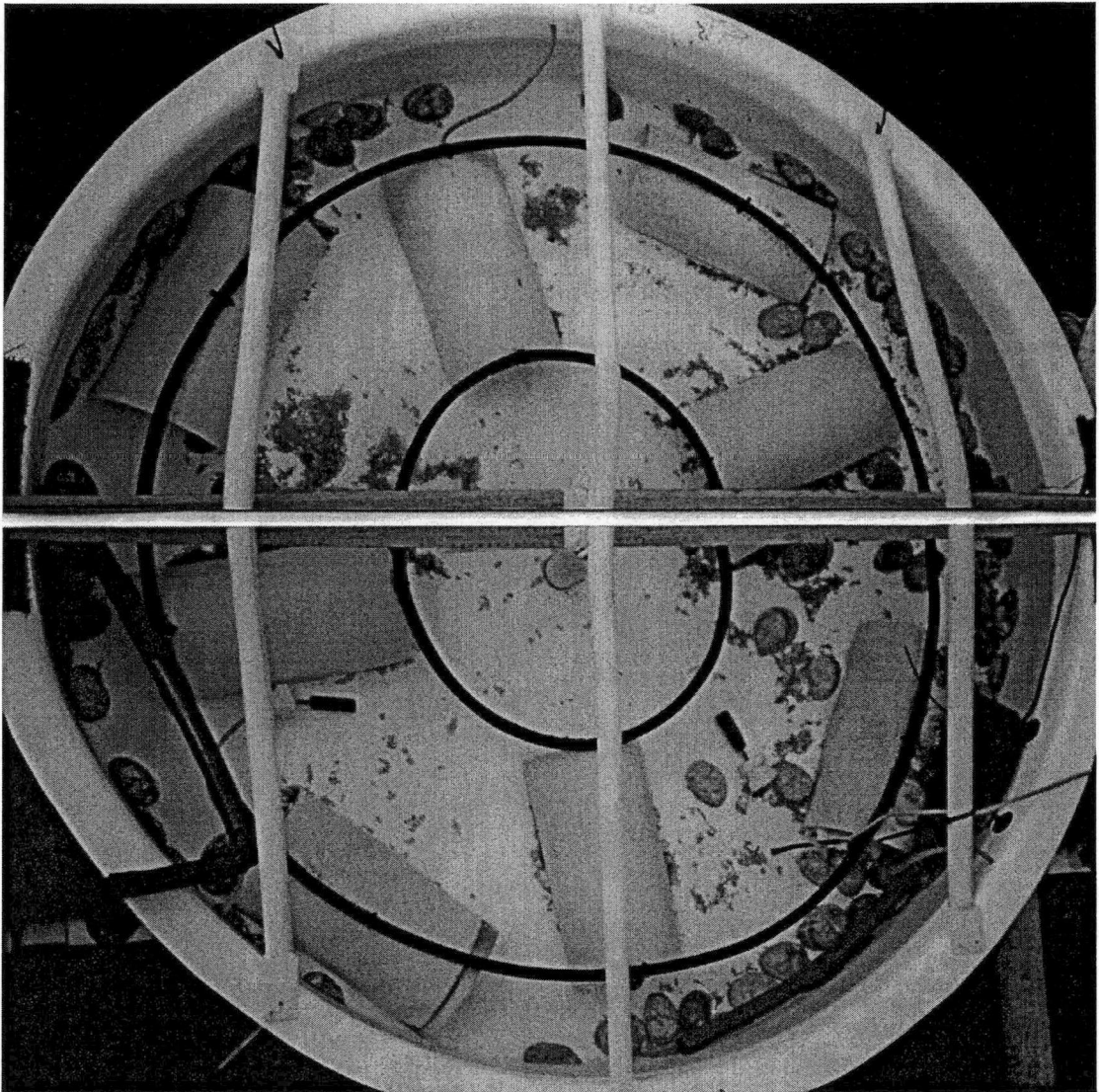


Plate 6.11 Treatment 6 during the day (240 abalone, 8 refuges). Notice the decrease in effective foraging area (EFA), compared to Plate 6.9. Fewer abalone are visible outside the refuges compared to Treatment 5 (Plate 6.9)

\_\_\_\_\_ = 30 cm



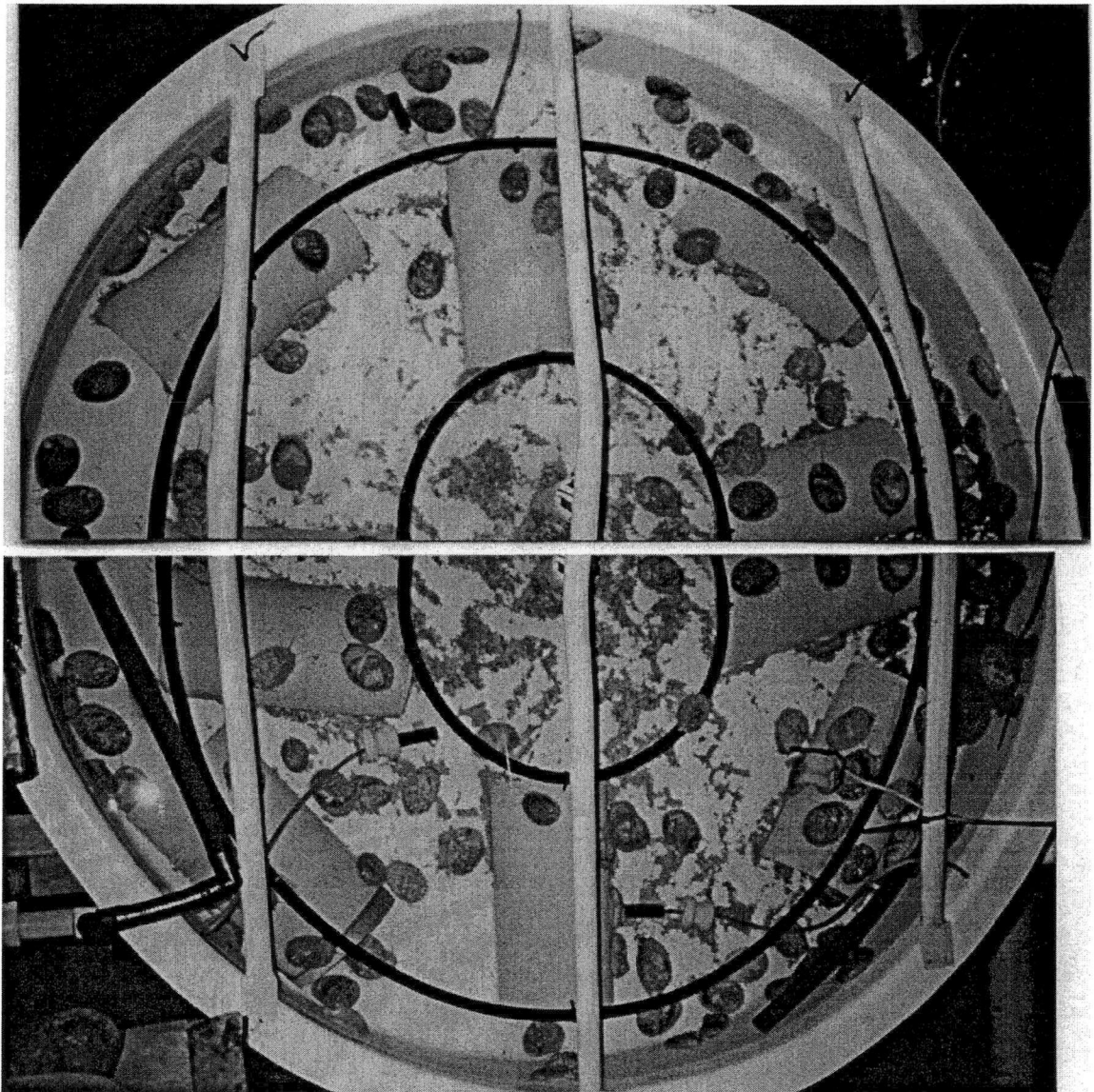


Plate 6.12 Treatment 6 at night. The effective foraging area can be clearly seen, and abalone are actively moving over all the available surfaces.

————— = 30 cm

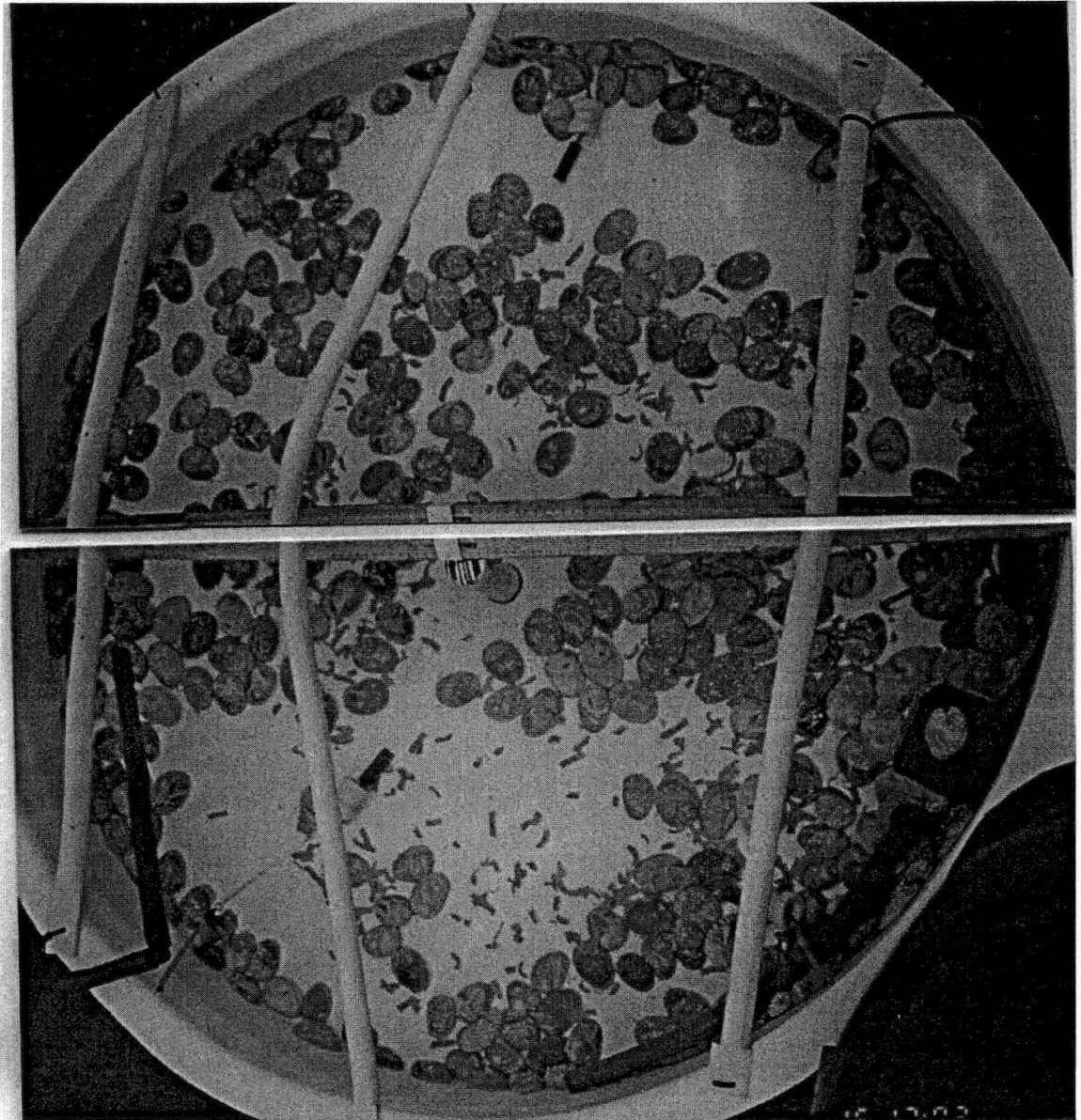


Plate 6.13 Treatment 7 during the day (360 abalone, no refuges). Abalone are gathered around the outer edges of the floor area. Organic wastes have accumulated between inactive abalone, which was not evident at lower densities (Plates 1, 7).

———— = 30 cm

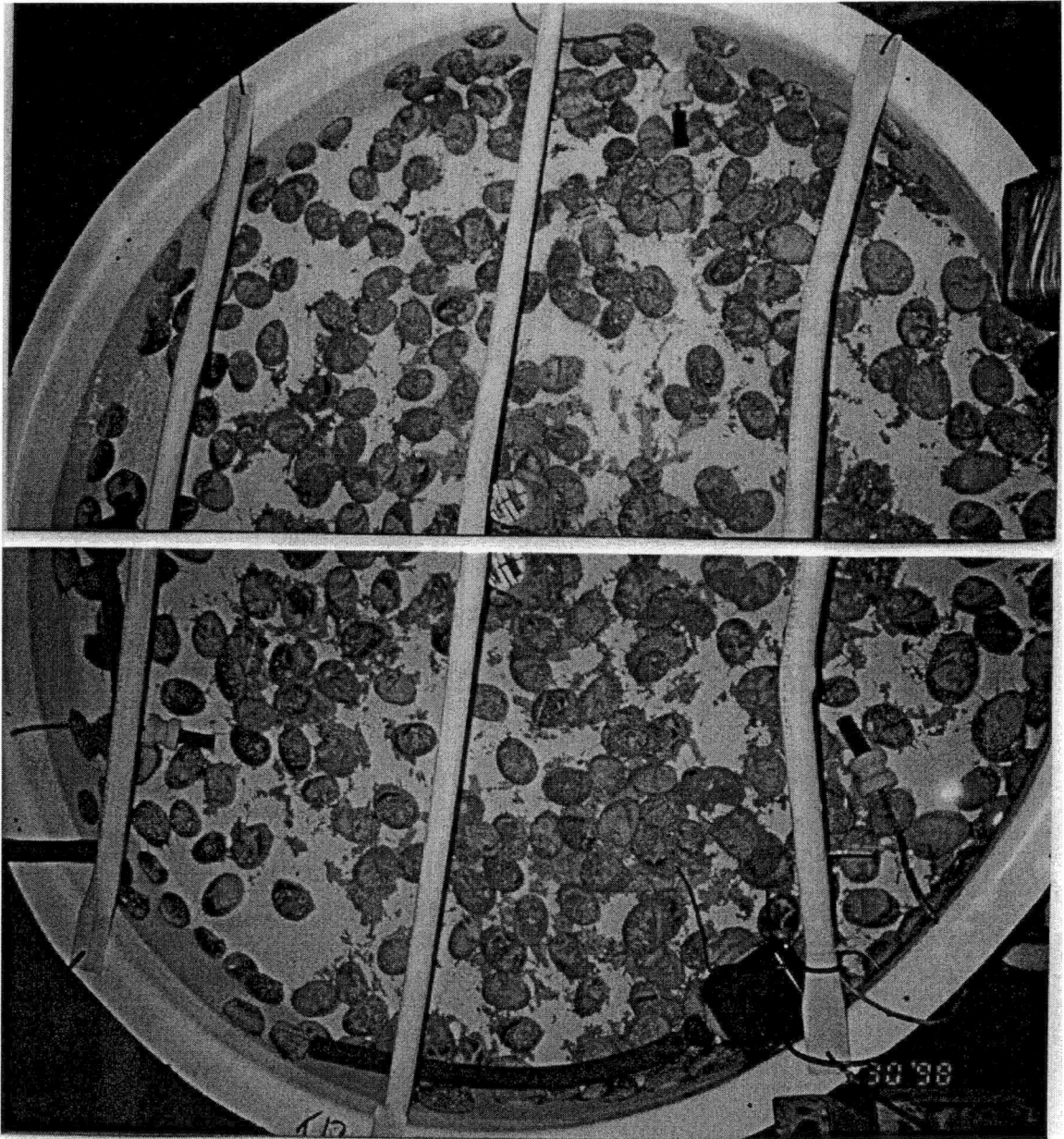


Plate 6.14 Treatment 7 at night. In the absence of refuges, there is a substantial decrease in EFA due to number of abalone on the floor of the tank. Abalone are clearly visible crawling over each other.

————— = 30 cm



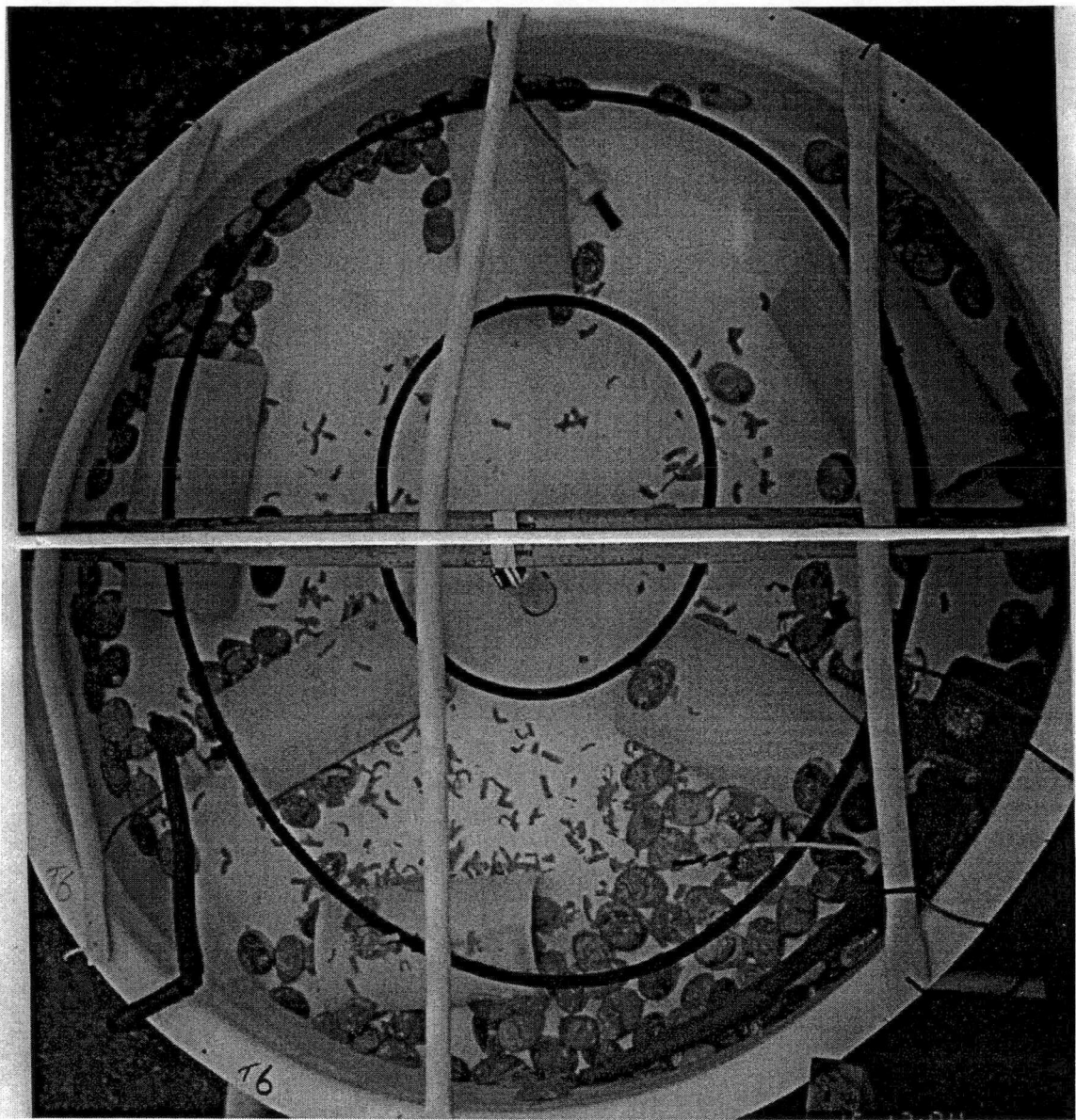


Plate 6.15 Treatment 8 during the day (360 abalone, 6 refuges). Abalone not under refuges are still around the outer areas of the tank, or along the edges of the refuges. Notice the accumulation of wastes around the refuges.

————— = 30 cm

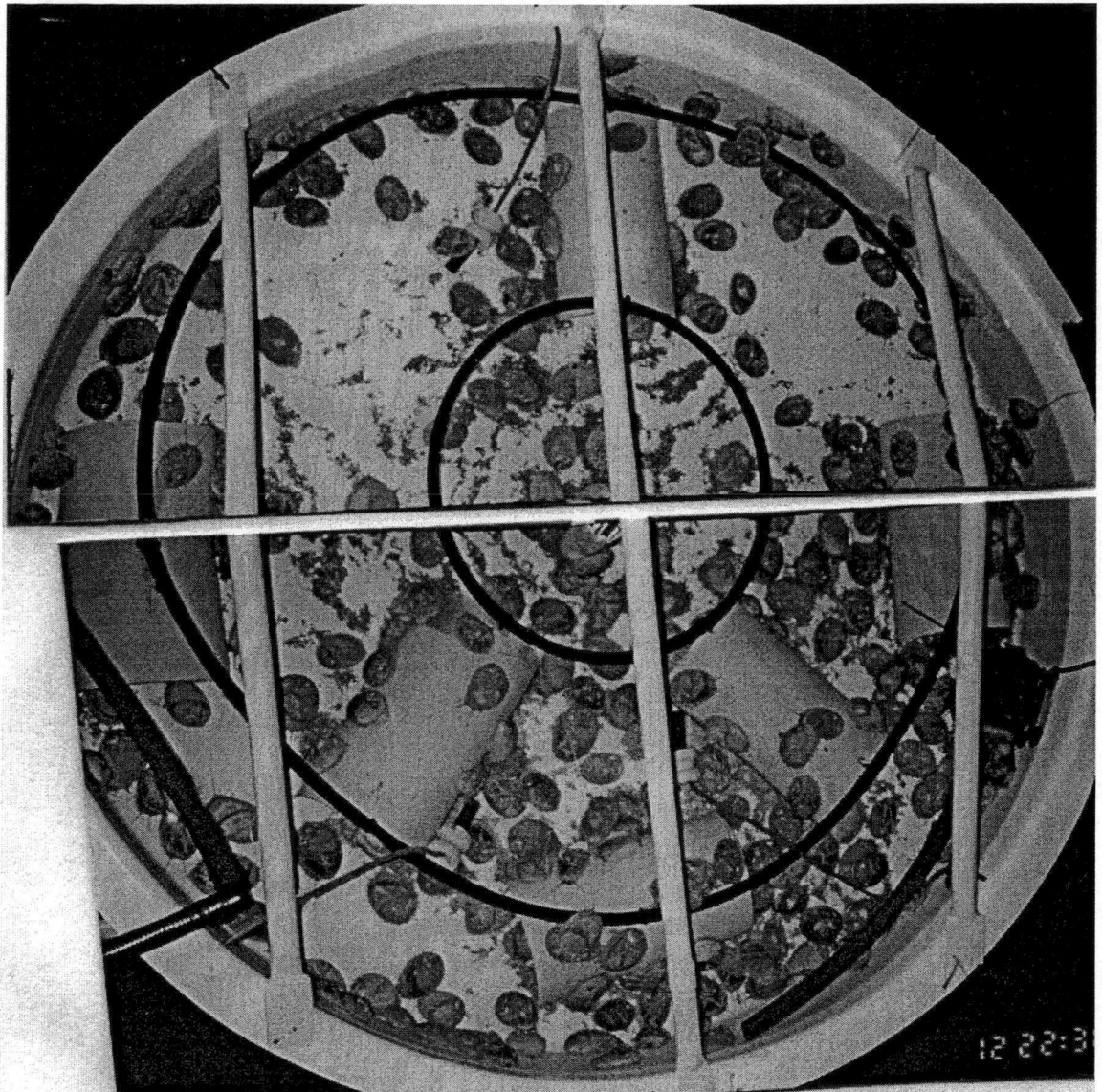


Plate 6.16 Treatment 8 at night. EFA is reduced by the number of refuges in the tank, and also by the foraging abalone. Abalone can also be seen pushing through accumulated wastes in the centre of the tank.

————— = 30 cm



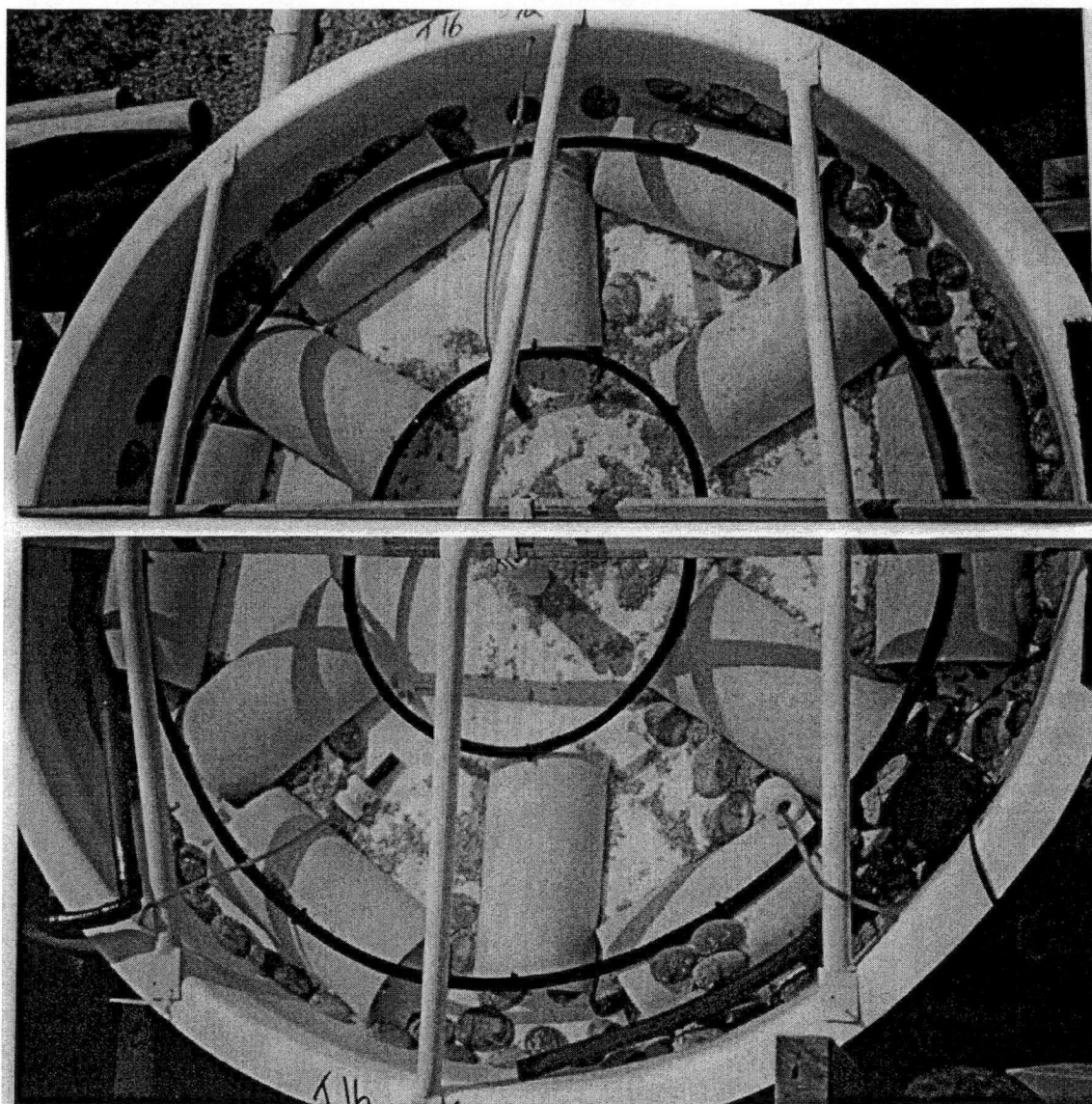


Plate 6.17 Treatment 9 during the day (360 abalone, 12 refuges). Fewer abalone are visible outside the refuges than in Treatment 8 (Plate 6.15), with those not under refuges being gathered around the outer edges of the tank.

————— = 30 cm

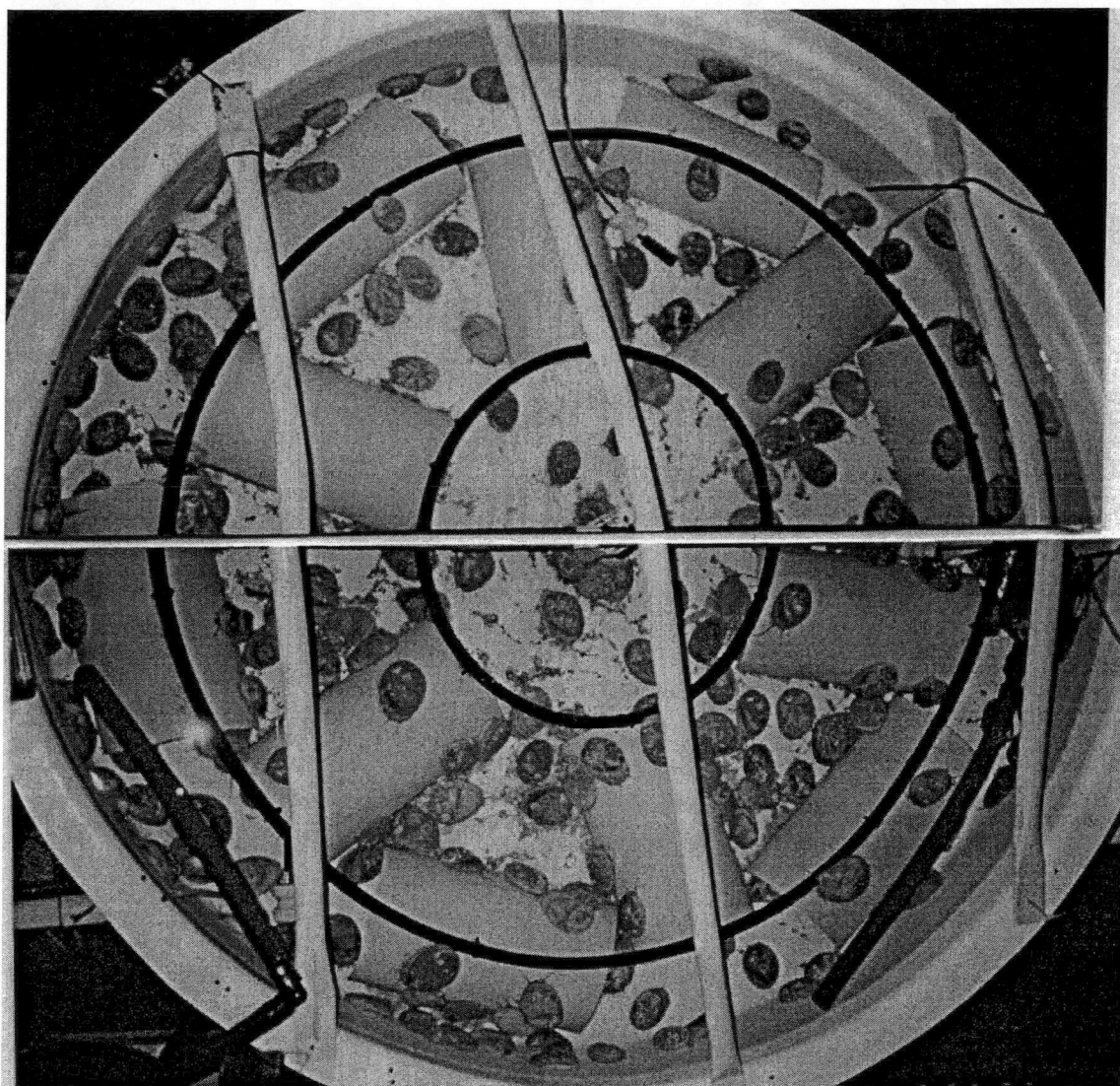


Plate 6.18 Treatment 9 at night. A further decrease in EFA compared to Treatment 8 is evident, due to the number of refuges and foraging abalone, although the submerged surface area (SSA) has increased.

————— = 30 cm

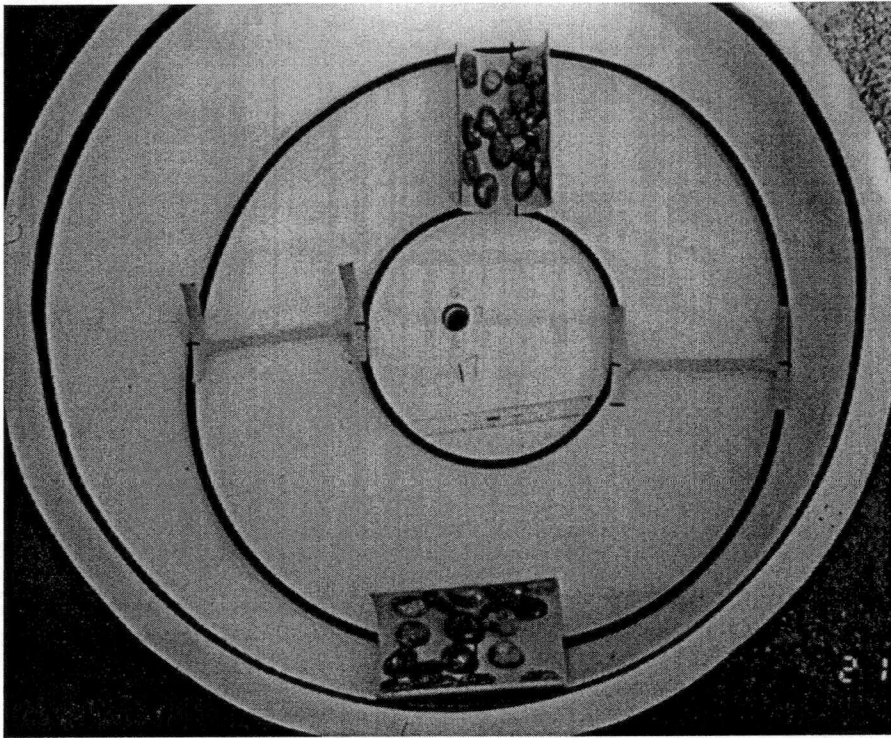


Plate 6.19 Refuges from Treatment 2 (120 abalone, 2 refuges/tank (1/60 abalone)).

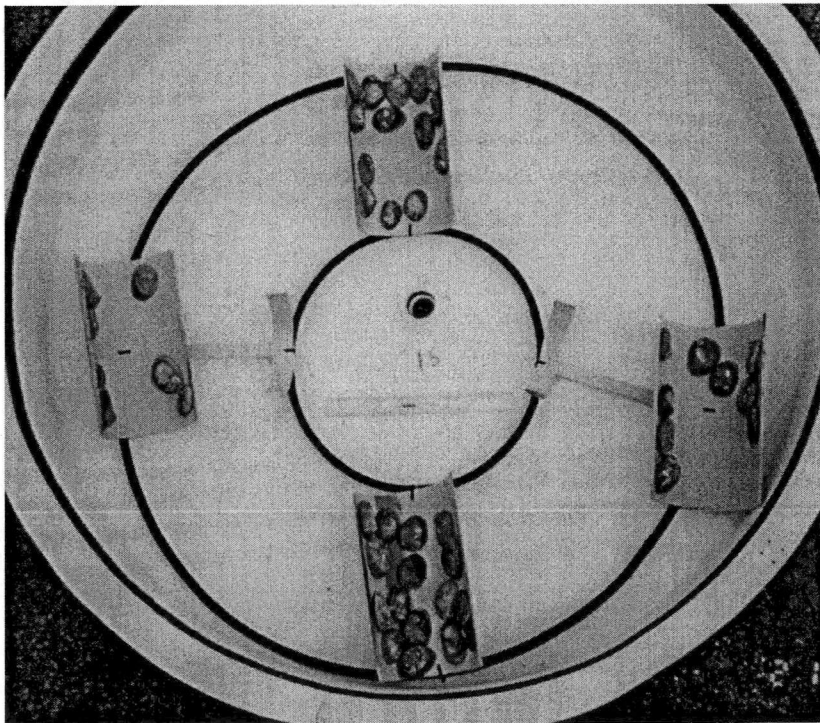


Plate 6.20 Refuges from Treatment 3 (120 abalone, 4 refuges (1/30 abalone)).

\_\_\_\_\_ = 30 cm



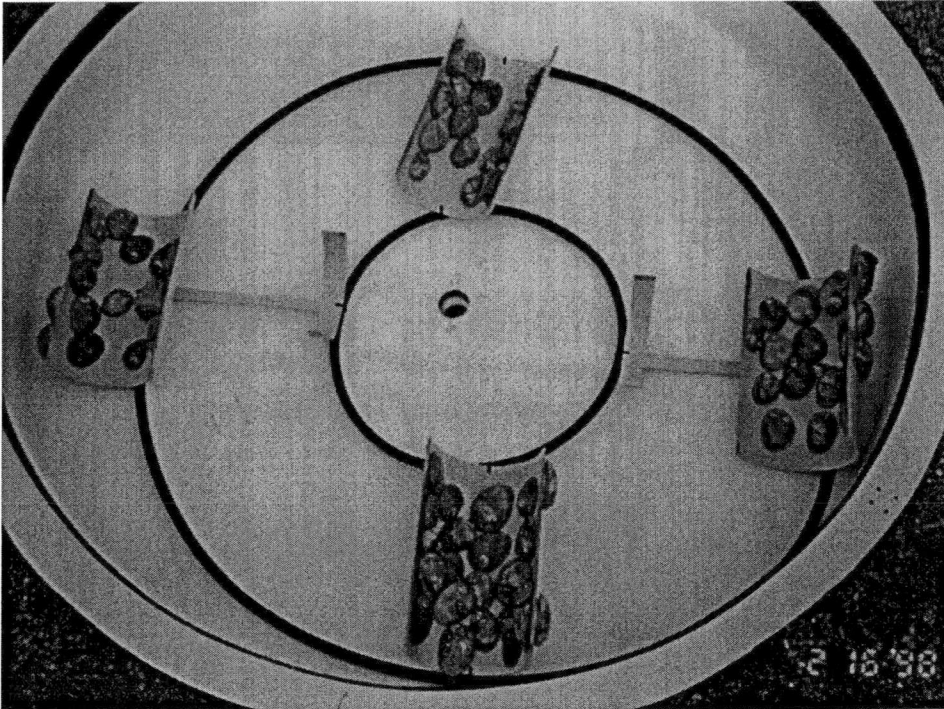


Plate 6.21 Refuges from Treatment 5 (240 abalone, 4 refuges (1/60 abalone)).

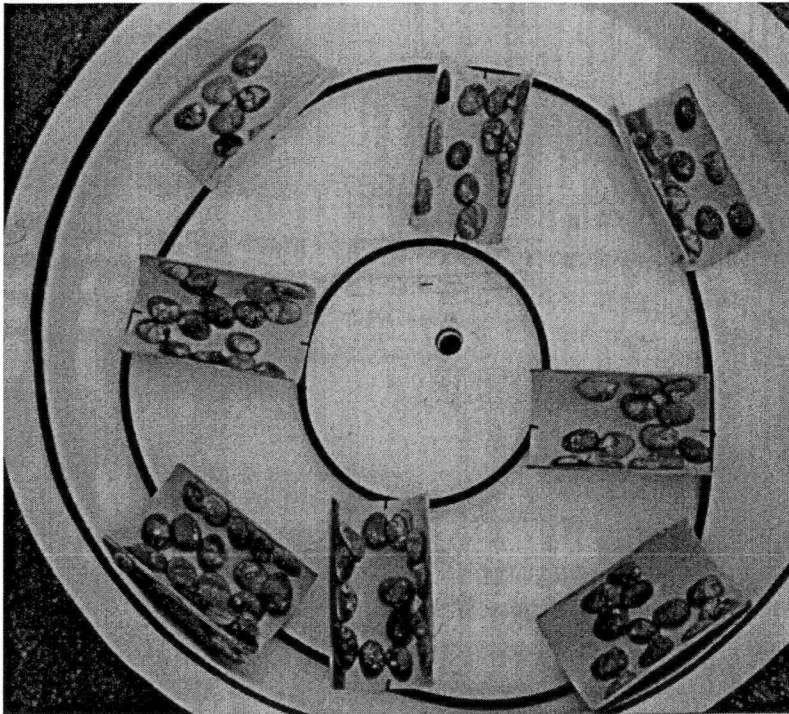


Plate 6.22 Refuges from Treatment 6 (240 abalone, 8 refuges (1/30 abalone)). Notice there are fewer abalone/refuge than for Treatment 5.

———— = 30 cm

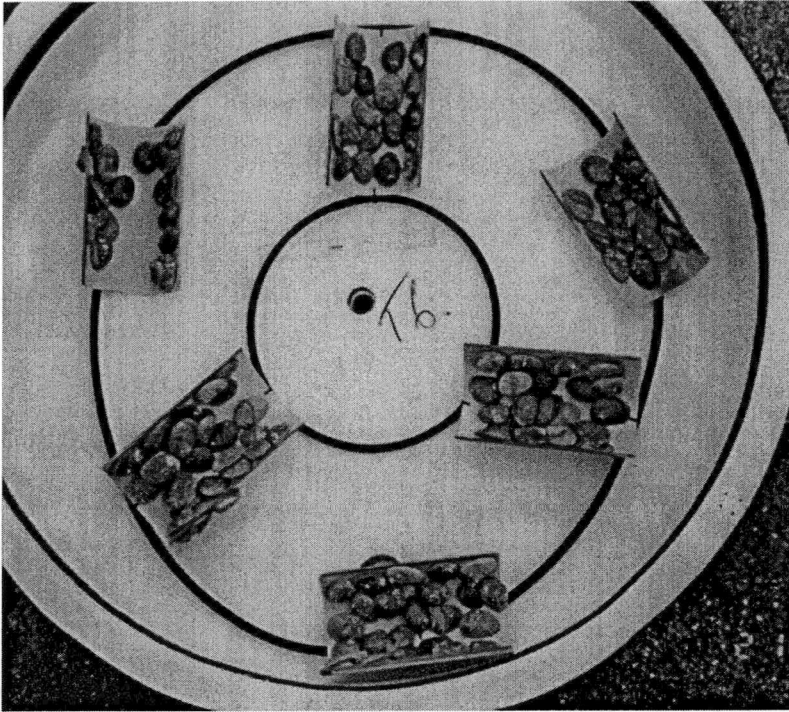


Plate 6.23 Refuges from Treatment 8 (360 abalone, 6 refuges (1/30 abalone)).

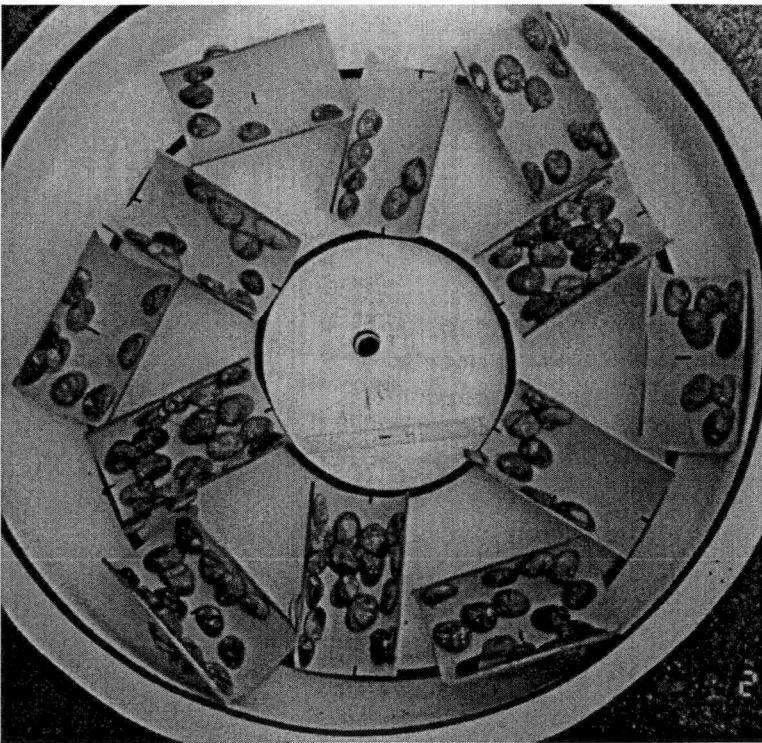


Plate 6.24 Refuges from Treatment 9 (360 abalone, 12 refuges (1/30 abalone)).  
\_\_\_\_\_ = 30 cm

## CHAPTER 7

### Temporal Changes In Water Quality

#### 7.1 Introduction

In order for the aquaculturist to maintain production and stock health at the high densities typical of intensive culture systems, an understanding is required of the factors that influence water quality in these systems. The high stocking densities generated in these systems have the potential to degrade water quality within the facility to the point that production is reduced or stock health is compromised (Porter *et al.* 1986; Millamena 1990; Poxton 1991; Allan *et al.* 1995). Reduced oxygen levels and accumulation of metabolites such as ammonia are commonly considered the most likely water quality variables to become problematic in intensive aquaculture (Porter *et al.* 1986; Millamena 1990; Poxton 1991). Under appropriate management, water exchange provides a continual dilution of metabolic wastes and replenishment of oxygen (Handy & Poxton 1993). However, once water exchange is reduced or interrupted, water quality becomes degraded due to metabolic activity of the standing stock.

Dissolved oxygen is considered a limiting factor for growth by compromising the capacity for aerobic metabolism rather than being directly toxic (Brett 1979), with low dissolved oxygen levels known to reduce growth in abalone (Harris *et al.* 1999a). Elevated environmental ammonia levels have more direct effects, disrupting a wide variety of biochemical processes in teleosts and molluscs (Sousa & Meade 1977; Chetty & Indira, 1995; Vedel *et al.* 1998). Chronic exposure to environmental ammonia has been shown to depress growth in abalone (Harris *et al.* 1998a; Basuyaux & Matthieu 1999). While intermittent exposure to elevated environmental ammonia combined with low dissolved oxygen levels had no effect on growth in either greenlip

or blacklip abalone (Chapter 2), chronic exposure to similar conditions resulted in significant growth depression in these species (Chapter 3). Chapter 3 also showed that abalone are more sensitive to elevated environmental ammonia in the presence of low dissolved oxygen than at >90% saturation.

Factors with the potential to influence water quality in abalone culture systems are shown in Figure 7.1. For land-based mariculture facilities the accumulation and subsequent degradation of organic-rich sediments composed primarily of faeces and uneaten food has the potential to influence water quality. Conditions within these wastes will differ substantially from the bulk water, with Roden (1997) demonstrating that this environment is reduced, acidic and sulphide enriched. Accumulated organic wastes have been shown to degrade water quality in larval prawn systems (Millamena 1990) and marine fish ponds (Porter *et al.* 1986). The effect of the noxious micro-environment within these wastes on bulk water quality will depend on the degree of transfer between the bulk water and the pore water within the wastes, which is essentially a function of the diffusive boundary layer (DBL) overlying the wastes (Allan *et al.* 1995). Benthic species such as abalone will be intimately associated with the DBL, and thus this micro-environment may be more important for these species than for freely swimming species such as salmonids.

Abalone are nocturnally active gastropods (Donovan & Carefoot 1998; Nakamura & Archdale 2001), being quiescent during the day and foraging for food at night. It would not be un-expected that this change in activity level may also contribute to alterations in water quality, and various studies have shown a nocturnal increase in oxygen consumption for several species of abalone (Jan *et al.* 1981; Peck *et al.* 1987; McBride *et al.* 2001).

Diet composition is an abiotic factor which also has the potential to influence water quality, but has not received much attention in the literature. Terrestrial plant ingredients are becoming common in formulated diets for many aquaculture species (Allan *et al.* 2000; Thiessen *et al.* 2003) including abalone (Britz 1996a; Fleming *et al.* 1996; Fleming *et al.* 1998; Shipton & Britz 2001). Abalone are known to be capable of degrading the complex polysaccharides found in algae (Fleming *et al.*

1996; Vandeppeer *et al.* 2002a), and some studies have shown that extracts from abalone digestive gland can degrade cellulose preparations *in vitro* (reviewed in Monjie & Viana 1998; Vandeppeer *et al.* 2002a). However, the endogenous capacity of halotids to break down terrestrial polymers from products such as pea and lupin meals in formulated diets is open to question (Monjie & Viana 1998; Vandeppeer *et al.* 2002a). Incorporating terrestrial ingredients in abalone diets may therefore lead to large amounts of less digestible material accumulating in the wastes, which could influence microbial degradation and increase substrate available for microbial colonization (Roden 1997). Diets with high levels of less digestible inorganic material (ie high in ash content) may also influence water quality by affecting microbial degradation of the wastes (Roden 1997). Excessive amounts of highly digestible protein sources such as fish meal may affect water quality by influencing ammonia excretion or microbial degradation of the accumulated wastes.

The aim of this trial was to investigate the factors shown in Figure 7.1, that may influence water quality in abalone grow out tanks. Superimposed on the effect of abalone metabolism during the day (resting) and at night (active) is the potential for the noxious mini-environment known to exist in accumulated wastes to influence bulk water quality (resting+wastes, active+wastes). Using a whole-tank approach, a sampling protocol was developed that investigated short term (< 24 hour) and longer term (5 day) effects of whole-tank metabolism on bulk water quality. An indirect method was used to assess pore water quality within the organic wastes. The influence of diet composition on bulk water quality and pore water was investigated by incorporating test ingredients into a control diet. The test ingredients consisted of an indigestible organic component (ground rice hulls), an indigestible inorganic component (kaolin) and a digestible organic component (fish meal).

## 7.2 Methods & Materials

The experiment was conducted at the same facility described in Chapter 2, with the same water supply. The experimental animals were cultured greenlip abalone



(approx. 3 years old), and prior to the trial they were held in a single commercial grow-out tank and fed a combination of formulated and natural diets.

The abalone were anaesthetised in benzocaine as per Hahn (1989d) and allocated to the experimental tanks in groups of 10. A total of 140 abalone (approx. 20 kg biomass.m<sup>-3</sup>) were allocated to each experimental tank, which were circular fibreglass units (diameter = 70 cm, volume = 55 L), with triplicate tanks allocated to each treatment. The experimental tanks used in the trial were enclosed within a heavily shaded area to prevent the development of algal biofilms. Animals from 8 of the 12 tanks used in the trial were weighed and measured at the end of the ten-week trial, to give an average length and weight for the experimental animals (8.17±2.84 g, 39±3.4 mm, means±SD, n=1009).

The influent water was heated to 16 °C, using an in-line heater in conjunction with a titanium plate heat exchanger which pre-heated influent water using the heated effluent from the experimental tanks.

The tanks were cleaned by siphoning the organic wastes every 4 days. Fresh food was presented every 2 days, at 1% of the estimated biomass in each tank.

### ***7.2.1 Experimental diets***

All diets were produced on site using a proprietary formulation based on semolina and defatted soya flour, with the combined ingredients being extruded using a commercial pasta maker and dried at 50 °C for 48 hr. There was one control diet and three experimental diets, with triplicate tanks on each diet. The experimental diets consisted of 85% control diet and 15% test ingredient. Rice hulls (certified as pesticide free) ground to a fine powder were used as a source of indigestible organic material. Technical grade kaolin was used as an indigestible inorganic component. Tasmanian fishmeal, based on jack mackerel, was supplied as a digestible nitrogen source.

### 7.2.2 Water quality

Dissolved oxygen (DO) was recorded in situ with a YSI probe and TPS meter (WP-82Y), calibrated against saturated sea water and Winkler titrations. pH was recorded in situ with a TPS meter and probe (WP-81), calibrated daily in fresh buffers. Samples for total ammonia (TA) were collected in acid-washed bottles, filtered through GF/C filters, and frozen for subsequent analysis in acid-washed glass or polyethylene bottles. TA was measured within 3 weeks using the basic method of Bower & Holm-Hansen (1980), but replacing the alkaline and oxidizing reagents with those given in Solorzano (1969). Un-ionised ammonia (UIA) was calculated from TA using the equation given in Bower & Bidwell (1978), in conjunction with pH and temperature data recorded at the time of sampling.

### 7.2.3 Experimental Protocol

The protocol was based on sampling bulk water quality (BWQ) at 4 different points in the normal cleaning cycle, with and without normal water exchange (T=0 & T=1 respectively), and is shown in Figure 7.2. Timing of the night-time water quality readings was intended to coincide with maximum foraging behaviour, based on personal observation of this species and other studies on a range of species (Hahn 1989d; Knauer *et al.* 1995; Nakamura & Archdale 2001). Measures were taken to minimize any disturbance of the normal nocturnal activity (use of low level red light, no physical disturbance of the tanks or stands).

The abalone were acclimatized to the experimental system and diets for 3–4 weeks before starting the sampling protocol shown in Figure 7.2, which was repeated for two complete cleaning cycles, 4 days apart. The tanks were cleaned by siphoning accumulated organic wastes and uneaten food every 4 days and fresh ration was provided every 2 days. If Day 4 of the old cleaning cycle is taken as Day 0 of the next cleaning cycle, each cleaning cycle covered 5 days. The results for each replicate tank were averaged to provide a mean value for individual replicates for statistical analysis ( $n=3$  for each diet). Disturbance of normal nocturnal activity of the abalone during the night-time sampling was minimised by using low-level red light and avoiding physical contact with the tanks.

*Bulk water quality (BWQ) under normal flow conditions (T=0)*

This involved sampling the bulk water quality for dissolved oxygen (DO), pH and total ammonia (TA) under normal water exchange conditions ( $T = 0$  in Figure 7.2). This sampling was carried out during the day and also at night when the tanks were free of organic waste on Day 1 (D1/Day, D1/Night in Figure 7.2), and on Day 5 when organic waste had accumulated (D5/Day, D5/Night in Figure 7.2). The tanks were cleaned between 09.00 – 10.00 on Day 1, with  $T = 0$  sampling being done between 13.00 – 15.00. Night-time sampling for  $T = 0$  was done between 22.00–24.00. Fresh ration was provided at 16.00–17.00 on Day 1 and Day 3.

*BWQ after an interruption to water exchange (T=1, whole tank metabolism)*

The effects of whole-tank metabolism on BWQ after an interruption to water exchange were investigated by interrupting water exchange for 60–90 minutes, and repeating the water quality measurements given above ( $T = 1$  in Figure 7.2). Six of the 12 experimental tanks were sampled again at 60 minutes, the remaining six at 90 minutes. The difference was due to the time it took to process the first lot of samples, but at least one replicate tank for each treatment was sampled at each time. Influent water was restored to all tanks at 90 minutes, immediately after sampling the second group of tanks. Aeration was maintained during the interruption to the water supply. For statistical analysis, changes in water quality were calculated on a per minute basis, then adjusted to 60 minutes.

*Sediment-enriched bulk water (SEBW)*

On Day 6, pore water quality within the wastes was assessed by siphoning 60–80% of the wastes into a 500 mL glass bottle and analysing this sample of sediment-enriched bulk water (SEBW). DO and pH were recorded within 10 minutes of the sample being collected. A sample for ammonia analysis was taken after the solids had been allowed to settle for 30–40 minutes, and was then treated as for ammonia

samples from the bulk water. Immediately prior to sampling SEBW, BWQ was recorded as for T=0.

#### ***7.2.4 Statistical Analysis***

Residual plots generated by JMP 3.2 (SAS Institute) were used to assess the data for homogeneity of variance. No transformations were necessary. Diet composition was compared using 1-way ANOVA and Tukeys HSD, using SPSS 10.02 (SPSS Inc.). Repeated measures ANOVA calculated by SPSS was used to determine whether there was a significant interaction between diet and time for BWQ, or diet and sample type for BWQ/SEBW comparisons on Day 6. There were no significant interactions ( $P>0.05$ ) for either T=0 or T=1 data for Day 1 or Day 5, so these data were pooled for diet ( $n=12$ ), and compared using Tukeys HSD. A Bonferroni correction ( $= 0.05/(\text{degrees of freedom})$ ) was used to adjust the Type I error rate for the multiple paired comparisons ( $P=0.083$ ) (Sokal & Rohlf 1995).

There was a significant interaction between diet and sample (BWQ or SEBW) type for the Day 6 (2.605, 3,  $P=0.088$ ), which was not evident when the results for the fish meal diet were removed from the pooled data set ( $P>0.05$ ). Data were pooled for the three other diets ( $n=9$ ), and compared with data from fish meal diet ( $n=3$ ) using ANOVA (SPSS).

### **7.3 Results**

The lack of any significant interaction between diet type (control, kaolin, rice hulls or fishmeal) and sampling time (Day 1 or Day 5, Night or Day) allowed the data to be pooled for diet and analysed as 12 replicates on one treatment rather than 3 replicates on 4 treatments, which provided much greater statistical power.

#### ***7.3.1 Diet Composition***

Data on diet composition are given in Table 7.1. The fish meal diet was significantly higher in nitrogen (186.98, 3, 4,  $P<0.001$ ) and carbon (18.481, 3, 4,  $P<0.001$ ) than the other three diets, and the kaolin diet was significantly lower in carbon ( $P<0.05$ ). The ash content of the kaolin diet was significantly higher than the

other diets, with ash content of the fibre and fishmeal diets similar and significantly higher than the control diet (5287, 3, 4,  $P<0.001$ ).

### ***7.3.2 Mortality Data***

Except for one event, mortality was virtually non-existent throughout the trial ( $<1\%$ ). The event involved one of the replicate tanks on the fishmeal diet, when the animals in that tank stopped eating, gathered around the air/water interface and there was 5-10% mortality. This tank and the abalone in it were removed from the trial, and returned to the control diet. On being transferred to a clean tank and different diet, mortality ceased and the animals recovered. This event occurred after the sampling reported here was completed.

### ***7.3.3 Temperature data***

As there was no influent water flow for 60-90 minutes before sampling at  $T=1$ , temperature changed slightly from that recorded at  $T=0$  (increasing during the day by 1-1.5 °C, and decreasing at night by 1-1.5 °C) for both sampling periods.

### ***7.3.4 BWQ during normal water exchange ( $T=0$ )***

Although the influent water and bulk water were not compared directly, Figure 7.3 shows that both DO and pH were lower than the influent water at all sampling points, with the difference increasing over the cleaning cycle. DO was significantly lower at D5/Day (resting+wastes) compared to D1/Day (resting), and D5/Night (active+wastes) compared to D1/Night (active) (149.98, 3, 44,  $P<0.001$ ). DO in the bulk water was significantly lower at D1/Night compared to D1/Day, but did not differ between D5/Day and D5/Night.

pH declined at each sampling point, and was significantly lower at D5/Day compared to D1/Day, and D5/Night compared to D5/Day (67.390, 3, 44,  $P<0.001$ ). pH in the bulk water was significantly lower at night compared to the day at both D1 & D5.

TA levels in the bulk water declined through time, and were significantly lower at D5/Day compared to D1/Day (20.524, 3, 44,  $P<0.001$ ). While there was a significant decrease in TA between D1/Day and D1/Night, there was no difference between D5/Day and D5/night.

### ***7.3.5 Effect on BWQ after an interruption in water exchange ( $T=1$ , whole tank metabolism)***

Figure 7.3 also shows the absolute changes in water quality in the absence of water exchange. Aeration was still provided during this time, which would have prevented oxygen levels falling as low as they otherwise would have done. During the day, temperature tended to increase during this time, but would not have had any substantial effect on the variables measured.

Figure 7.4 shows the data calculated as rates of change (consumption or production). Despite the significant difference in DO levels between D1/Day (resting) and D1/Night (active) at  $T=0$  (Fig. 7.3), oxygen consumption did not differ significantly at these times. On Day 5, the rate of oxygen consumption was lower during the day than at night, but the difference was not statistically significant. Although oxygen consumption at D5/Day was significantly lower than at D1/Day (10.540, 3, 44,  $P<0.001$ ), it did not differ between D5/Night (active+wastes) and D1/Night (active).

pH declined during the interruption in water flow for both Day 1 and Day 5, but the extent of the change differed significantly between the day-time and night-time readings (158.084, 3, 44,  $P<0.001$ ). On Day 1, the decline was significantly higher during the night than it was during the day. At Day 5, the decline in pH was significantly higher during the day than it was at night. The decrease in pH was significantly higher at Day 5/Day (resting+wastes) than for Day 1/Day (resting), but did not differ between Day 1/Night (active) and Day 5/Night (active+wastes).

For both Day 1 & Day 5, the increase in TA was higher during the day than at night, but only differed significantly on Day 5 (5.480, 3, 44,  $P<0.001$ ).

### 7.3.6 SEBW

Figure 7.6 indicates that the pore water within the organic wastes is oxygen depleted, acidic and contained substantially higher levels of TA than the bulk water.

2 way ANOVA showed that diet had a significant effect (2.605, 3,  $P=0.088$ ) Pooling the data for control, kaolin and fibre diets (which did not differ significantly from each other ( $P>0.05$ )) and comparing this with SEBW from the fishmeal diet showed that the fishmeal diet produced organic wastes which were significantly higher in TA (37.118, 1, 10,  $P<0.001$ ), lower in pH (8.823, 1, 10,  $P=0.014$ ) and lower in DO (7.042, 1, 10,  $P=0.024$ ). These differences were not apparent in the BWQ data.

## 7.4 Discussion

Mortality was low throughout the trial, except for the single event mentioned above. This episode occurred in one of the replicates on the fish meal diet, which had the highest nitrogen content of the diets used in this trial. It is possible that the nitrogen content of this diet encouraged bacterial growth which predisposed this tank to an opportunistic infection. This is also supported by the fact that mortality ceased once the abalone were transferred to a clean tank and the diet was changed to the control diet.

The lipid solubility of neutral molecules such as  $\text{NH}_3$  and the predominance of un-ionized ammonia in most aquatic environments has traditionally implicated this form of ammonia as the primary toxic agent (Bower & Bidwell 1978; Wilkie 1987). However, it is becoming increasingly evident that the leaky paracellular junctions in branchial tissue of marine organisms are more permeable to cations such as  $\text{NH}_4^+$  than previously considered (Wilson & Taylor 1992; Wilkie 1997), and the results are therefore discussed in terms of TA rather than UIA.

This trial considered the impact of two components shown in Figure 7.1 on water quality – abalone metabolism and accumulated organic wastes. Other components such biofilm respiration were considered unlikely to have sufficient biomass compared to either abalone or organic wastes to have any substantial impact. Comparing samples taken during the day (resting) with those taken at night (active) allowed the effect of resting metabolism to be compared with the increase due to

foraging activity. Comparing these results with those on Day 5, when organic wastes had accumulated through the cleaning cycle, allowed any impact of these wastes on BWQ or abalone metabolism to be determined. On Day 6 conditions inside the organic wastes were assessed, and substantial differences to the BWQ were revealed.

#### **7.4.1 BWQ ( $T=0$ & whole tank metabolism)**

Both nocturnal foraging activity and accumulation of organic wastes were shown to have modest but statistically significant impacts on BWQ during normal water exchange.

Oxygen levels in the bulk water at  $T=0$  did not differ between day and night at Day 5, probably due to the fact that water exchange from the influent water and the supplementary aeration were sufficient to cover any deficit due to foraging activity. Oxygen consumption increased by 30% at Day 5/Night (active+wastes) due to foraging activity. Various authors have reported an increase in nocturnal oxygen consumption in abalone (Jan *et al.* 1981; Peck *et al.* 1987; McBride *et al.* 2001), although the extent of the increase differs, being significant in some studies but not others. This may be due to the fact that on any given night not all abalone may be fully active, with a range of activity states having been described between fully quiescent and actively foraging (Donovan & Carefoot 1998; Nakamura & Archdale 2001). Most of the published studies are also based on single individuals or small numbers of abalone held in small respirometer chambers, sometimes fed and sometimes starved, which may have altered normal metabolism and foraging activity.

Nocturnal oxygen consumption did not differ from daytime oxygen consumption on Day 1. Figure 7.3 shows that there were substantial alterations in water quality at  $T=0$  by Day 5, compared to the influent water, and it is possible that the disturbance to the tank and sudden alteration in water quality after cleaning may induce a stress response which could increase oxygen consumption during the day. It is also possible that oxygen levels in the water column had not reached the normal equilibrium with the abalone biomass after cleaning. When the system is in equilibrium, the resting abalone biomass will consume a certain amount of oxygen



from the water column in the absence of organic wastes, but after the removal of 30-50% of the tank volume during cleaning this equilibrium will take time to become re-established. Calculations based on respirometry data generated from the same system and presented in Chapter 8 show that this equilibrium position should result in a baseline oxygen level at  $T=0$  of around  $7.3 \text{ mg.L}^{-1}$  during the day, while Figure 7.4 shows that the recorded DO was  $7.7 \text{ mg.L}^{-1}$ . This would explain why oxygen levels were significantly higher during the day than at night for Day 1, and would alter the oxygen consumption measured at Day 1/Day (resting) from  $0.8$  to  $0.5 \text{ mg}^{-1}.\text{L}^{-1}.\text{hr}^{-1}.\text{tank}^{-1}$ . These changes would result in a similar pattern to Day 5, with oxygen levels similar at  $T=0$  for both day and night, but with oxygen consumption lower during the day.

The significant decrease in pH in the bulkwater between Day 1/Day (resting) and Day 5/Day (resting +wastes) is due to the accumulation of organic wastes, which would also explain the decrease between Day 1/Night (active) and Day 5/Night (active+wastes). The decrease in bulkwater pH between day and night observed at both Day 1 and Day 5 is the result of increased metabolic activity due to foraging. The change in pH was similar for both Day 1/Night (active) and Day 5/Night (active+wastes), but differed between Day 1/Day (resting) and Day 5/Day (resting+wastes). The small change at Day 1/Day compared to Day 5/Day may be due to tank cleaning having some effect on abalone metabolism or masking the normal effect of abalone metabolism, as for DO. The larger change in pH at Day 5/Day compared to Day 5/Night is probably due to post-prandial metabolism increasing the release of acid equivalents. Removal of a percentage of the tank volume during cleaning may have removed these equivalents, and prevented the effect being detected.

Ammonia levels in the bulk water declined over the cleaning cycle as for pH. It is possible that the higher level of TA found at Day 1/Day is due to disturbance of the organic wastes during cleaning releasing ammonia into the bulk water, which had not been diluted to normal background levels by the time samples were taken at  $T=0$ . As discussed in the next section the environment within the organic wastes is acidic, high

in ammonia and low in oxygen, and the diffusive boundary layer overlying these wastes could conceivably generate a micro-environment which stresses the abalone and reduces food consumption, and hence ammonia excretion. This may contribute to the reduction in oxygen consumption at Day 5/Day, and the lower level of TA in the bulk water at Day 5 compared to Day 1.

An increase in ammonia excretion during the day would be due to catabolism of protein from the ration consumed during foraging. This pattern of ammonia excretion is well established in teleosts, with peak excretion rates being detected 3-8 hours following feeding (Porter *et al.* 1987; Dosdat *et al.* 1996; Forsberg 1996, 1997), and has also been reported in gastropod molluscs (Crisp *et al.* 1981, cited in Bishop *et al.* 1983). The fact that the excretion rate did not differ between Day 1 and Day 5 indicates that abalone metabolism, at least in terms of ammonia excretion, was not affected by the presence of accumulated organic wastes.

Based on chronic bioassay in chapter 3 and Harris (1999), the levels of TA, DO or pH found in the bulk water either during normal water exchange or after 60 minutes with no water exchange (Figure 7.3, 7.4) are unlikely to reduce growth or increase mortality in greenlip abalone (Harris *et al.* 1998a, 1999a, b). Chapter 2 showed that intermittent exposure to conditions shown in Figure 7.3 following an interruption in water exchange would also not decrease growth. These results also show the importance of an adequate water exchange in maintaining oxygen levels and diluting metabolites. If water exchange is insufficient, water quality could be degraded to the point where growth and stock health will be compromised due to a combination of abalone metabolism and accumulated organic wastes.

#### **7.4.2 SEBW**

The indirect method used in this study to assess pore water quality (SEBW) produced results comparable with pore water analyses from other studies using conventional methods (Table 7.3). In a study based on micro-electrode measurements of pore water quality in undisturbed organic waste in abalone tanks, Roden (1997)

found that the pore water was acidic, low in dissolved oxygen and reduced, and that sulphide was present by Day 3 of a 5-day cleaning cycle.

Water quality data at T=0 indicate that there is a degree of transfer between the pore water and bulk water compartments but the effect is modest during normal water exchange. There is some evidence that the accumulated organic wastes may have imposed a stress on foraging abalone and reduced food consumption. Abalone bioturbating the wastes will be acutely exposed to severely altered water quality. Such exposures repeated over time may have a cumulative effect, which may be amplified if other stressors such as sub-optimal water quality are in effect. Exposure to the degraded environment within organic wastes has been implicated in depression in prawn growth from model farming ponds (Allan *et al.* 1995).

It should also be noted that abalone will be intimately associated with the diffusive boundary layer overlying the organic wastes, which will differ from BWQ. Ionised ammonia ( $\text{NH}_4^+$ ) may have a more important role in ammonia toxicity in this environment, as the low pH will tend to push the equilibrium toward this form rather than UIA. Ionised ammonia is potentially more toxic to marine species due to a greater membrane permeability to cations than for fresh water species (Wilkie 1997; Wilson & Taylor 1992). Sulphide has also been reported in wastes from abalone systems (Roden 1997) and marine fish ponds (Porter *et al.* 1986), and has been associated with mortality in abalone (Chen 1989).

#### **7.4.3 Effect of Diet Composition**

While there was no significant diet effect in terms of BWQ, the SEBW for the fish meal diet differed sufficiently from the other diets in terms of DO, pH and TA to produce a significant diet effect (Figure 7.5). These differences are indicative of a higher rate of microbial respiration causing a higher rate of ammonia production in the organic wastes from the fish meal compared to the other diets. Optimum protein levels for abalone diets range between 20-30% (Uki & Watanabe 1992; Fleming *et al.* 1996), while the fish meal diet had 39.5%. The excessive level of protein in the fish meal diet did not produce a significantly higher level of TA in the bulk water nor

increased ammonia production, indicating that excess protein rather than ammonia is excreted in the faeces. This is confirmed by significantly higher levels of nitrogen in the organic wastes (Chapter 8).

Abalone are known to be able to digest the structural polysaccharides in algae, which differ chemically from terrestrial polysaccharides such as cellulose (Fleming *et al.* 1996). While abalone are known to have the capacity to digest cellulose, it is not clear whether this is due to the activity of endogenous enzymes or bacterial flora in the digestive tract (Monjie & Viana 1998; Vandeppeer *et al.* 2002a). There is an increasing interest in the use of terrestrial ingredients in abalone diets (Britz 1996a; Fleming *et al.* 1996; Fleming *et al.* 1998) that contain substantial amounts of non-starch polysaccharides such as cellulose. Lack of significant effects due to inclusion of fibre or kaolin suggests that the increase in inert organic or inorganic material did not lead to an increase in microbial degradation of the wastes. This suggests that diets with relatively indigestible organic or inorganic fractions would not have a substantial impact on water quality, and would therefore be suitable for inclusion in abalone diets.

## 7.5 Conclusions

Temporal changes detected in the water quality parameters measured were primarily due to nocturnal foraging activity and post-prandial metabolism in the short term (<24 h), and the accumulation of organic wastes over the longer term (5 d). Significant differences in BWQ due to diet were not detected, but fish meal did produce significant differences in SEBW compared to the other diets. Analysis of SEBW indicates that the pore water within the organic wastes is oxygen depleted, acidic and high in ammonia. The impact on bulk water of the micro-environment within the wastes is modest as long as normal water exchange is maintained. Abalone disturbing the waste will be transiently exposed to a noxious environment.

The results indicate that excess protein is voided as faecal waste, which leads to a higher rate of microbial activity in the sediments. While the organic wastes do have a modest influence on DO and pH in the bulk water, abalone are the main factor

influencing water quality both during normal water exchange and when water exchange is interrupted.

Although TA levels declined through the cleaning cycle, ammonia production is higher during the day than at night.

Diet	Experimental Diet			
	Control	Kaolin	Fibre	Fish meal
% Nitrogen	4.84±0.24 <sup>b</sup>	4.13±0.16 <sup>b</sup>	4.59±0.39 <sup>b</sup>	6.32±0.38 <sup>a</sup>
% Carbon	38.95±0.91 <sup>b</sup>	32.00±0.85 <sup>c</sup>	37.00±0.43 <sup>b</sup>	47.3±0.42 <sup>a</sup>
% Ash	6.03±0.13 <sup>a</sup>	20.14±0.02 <sup>b</sup>	7.93±0.10 <sup>c</sup>	7.47±0.06 <sup>c</sup>

Table 7.1 Composition of control and experimental diets on a percentage dry matter basis (means ± SE, n=2). Test diets were 85% control diet, and 15% experimental ingredient (air dried basis). The control diet was based on a proprietary formulation, based on semolina and defatted soya flour. Using a conversion factor of 6.25, protein levels were 30.25%, 25.81%, 28.68% and 39.5% respectively. Different superscripts indicate significantly different means for individual components (P<0.05).

Comparison	Test	F	Degrees of Freedom	P
T = 0 (Figure 7.3)	Dissolved Oxygen	149.977	3, 44	0.000
	pH	67.390	3, 44	0.000
	Total Ammonia	20.524	3, 44	0.000
T = 1 (Figure 7.3)	Dissolved Oxygen	5.277	3, 44	0.003
	pH	193.534	3, 44	0.000
	Total Ammonia	22.011	3, 44	0.000
Change T=0>T=1 (Figure 7.4)	Dissolved Oxygen	10.540	3, 44	0.000
	pH	158.084	3, 44	0.000
	Total Ammonia	5.480	3, 44	0.000
Diet Composition	Nitrogen	186.979	3, 4	0.000
	Carbon	18.481	3, 4	0.008
	Ash	5287.756	3, 4	0.000

Table 7.2 Data from ANOVA. Due to a lack of any significant interaction between diet and time (using a repeated measure analysis in SPSS), these analyses were based on data pooled for diet (n=12).

Author	Source	Sample	Total Ammonia Nitrogen (mg.l <sup>-1</sup> )	pH	Dissolved Oxygen (mg.l <sup>-1</sup> )	Eh (mV)
Masuda & Boyd, 1994	freshwater fish ponds, Alabama, USA	Bulk water	0.30 - 0.29	8.3 - 8.7		240 - 252
		Pore water	2.17 - 21.64	6.86 - 6.9		-90 - -156
Martin <i>et al.</i> 1998	marine prawn ponds, New Caledonia	Pore water	1.25-6.84			
Roden 1997	abalone tanks	Bulk water		8.11 - 8.27	5.3 – 7.1	99.94 - 184.95
		Pore water		6.41 - 44	1.10 - 3.65	9.49 - -116.89
Present study	abalone tanks	Bulk water	0.08	7.93	7.07	
		Pore water	0.83	5.83	4.30	

Table 7.3 Comparison of literature values for pore water and sediment enriched bulk water (SEBW) from the present study. SEBW was determined by siphoning a sample of the organic wastes into a glass bottle and testing the supernatant for DO, pH and TA.



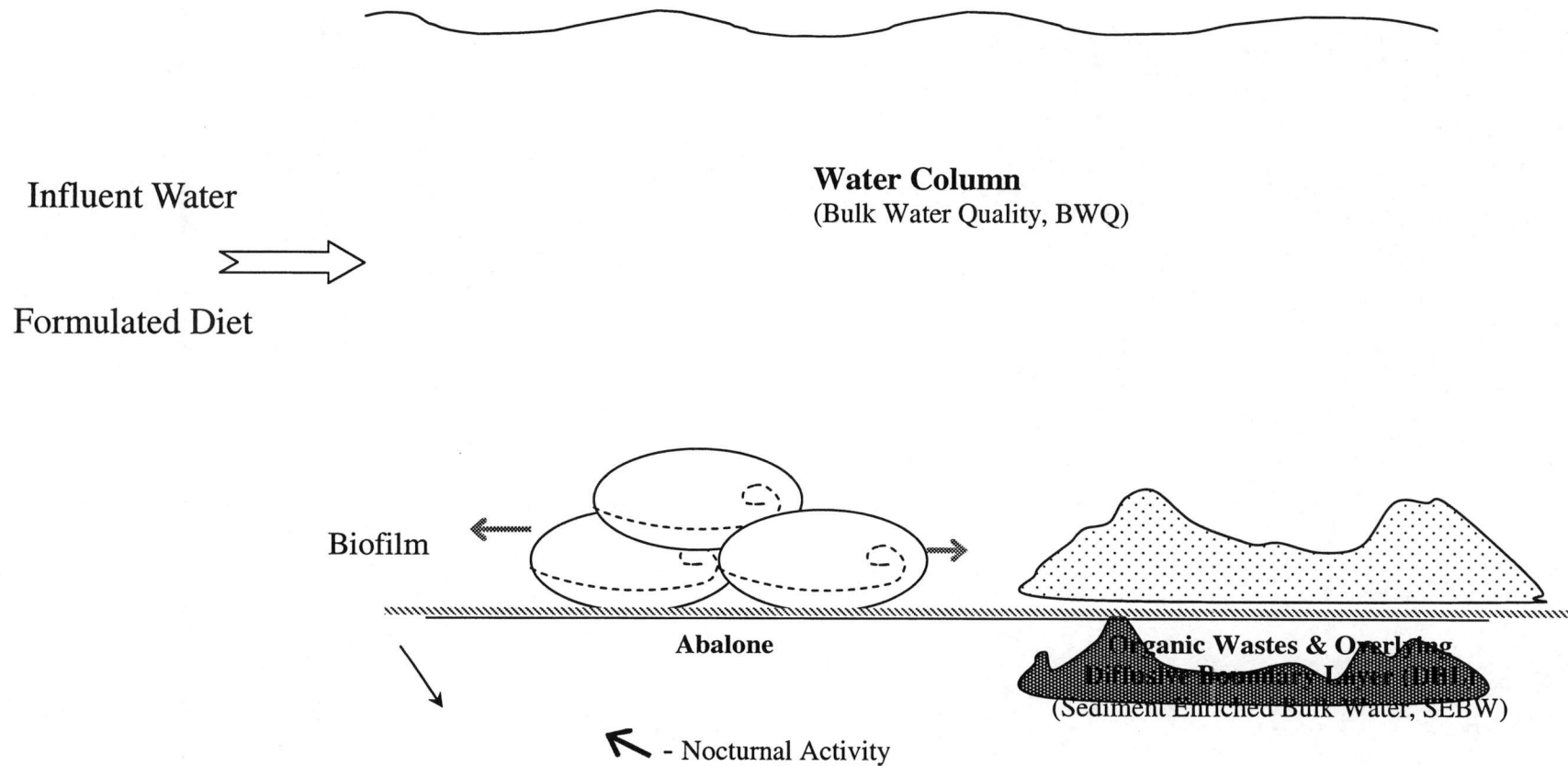


Figure 7.1 Components of a pilot scale abalone system which may impact on water quality. Given the relative size of any biofilm, compared to abalone biomass or organic wastes, it was assumed that biofilms would have very little if any impact on water quality in this study. The exclusion of light from the experimental system means that the biofilms would be primarily bacterial rather than algal in composition.

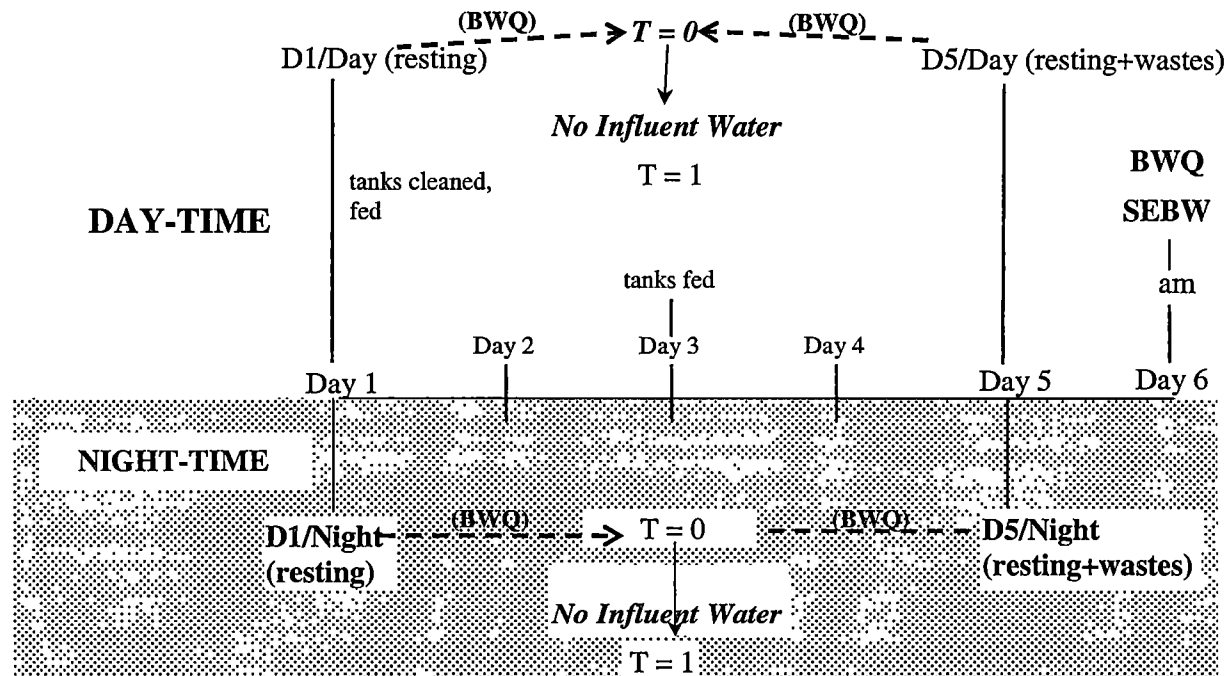


Figure 7.2 Sampling protocol and time-line for sampling Bulk Water Quality (BWQ) and Sediment Enriched Water Quality (SEBW). On Day 1, tanks were cleaned by siphon between 09.00 – 10.00, and BWQ recorded for  $T=0$  between 13.00 – 15.00. Water exchange was then halted for 60-90 minutes, and BWQ sampled again ( $T=1$ ) as for  $T=0$ . Aeration was maintained during the interruptions in water exchange. The tanks were fed the normal ration after  $T=1$  on Day 1, and on Day 3 (16.00-17.00). Night-time samples for  $T=0$  were taken between 22.00 – 24.00, and then the influent water flow was turned off and  $T=1$  samples taken as described above. On Day 6, BWQ was sampled as for  $T=0$ , and sediment-enriched bulk water (SEBW) as described in the text. After the sampling on Day 6, the tanks were cleaned and fed as on Day 1. After a full 5 day cleaning cycle, the sampling protocol was repeated again.

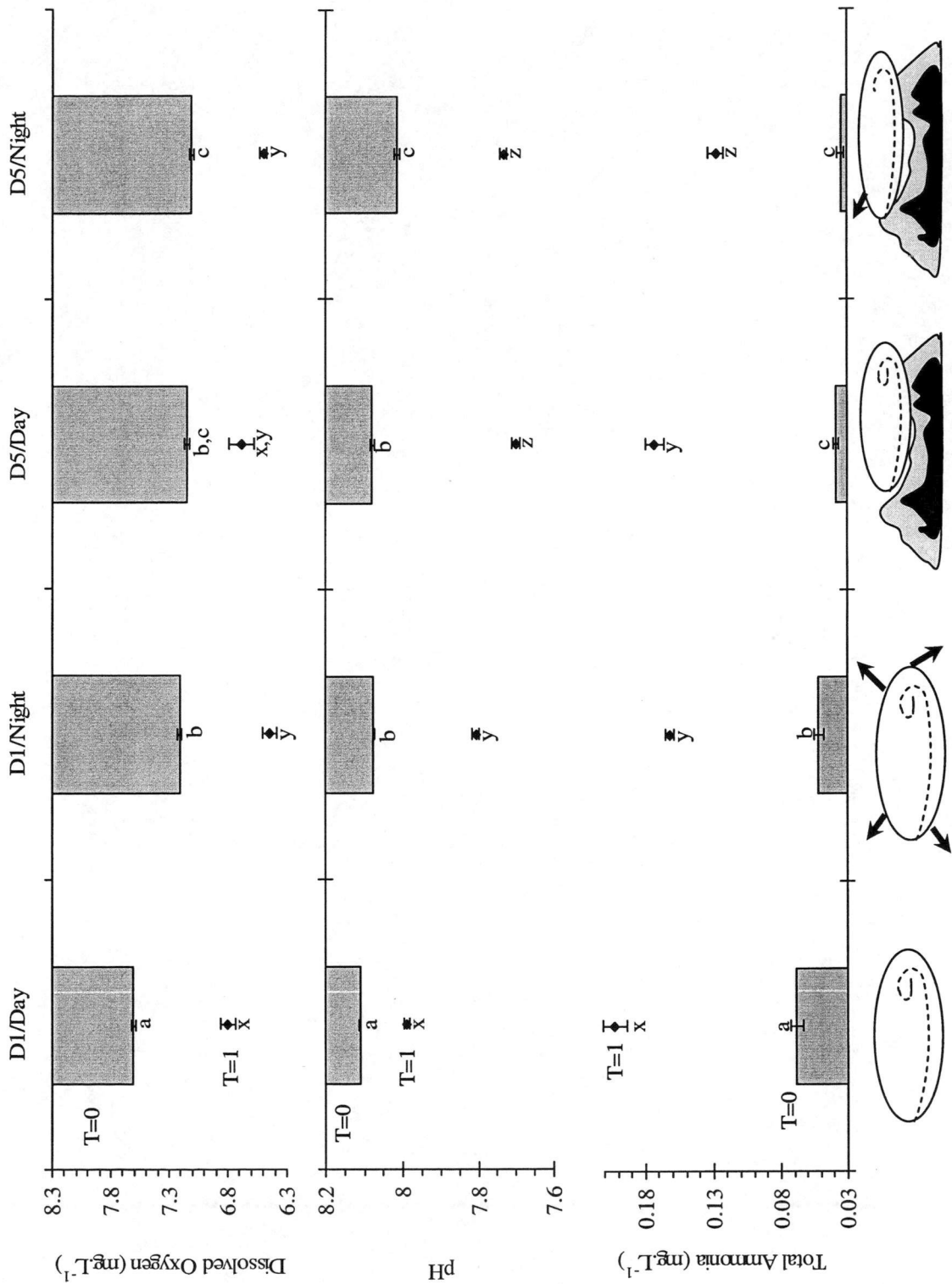


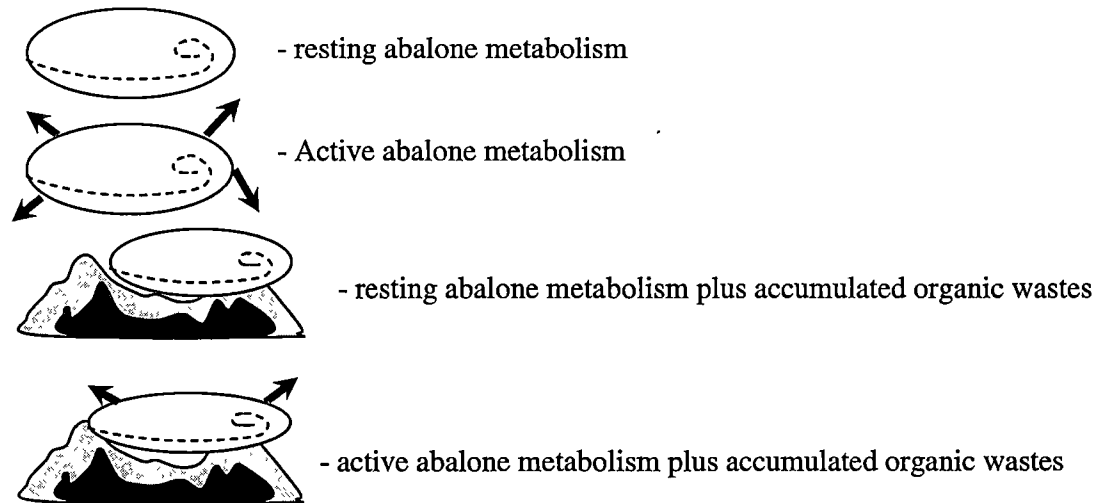
Figure 7.3 Bulk water quality (BWQ) at T=0 & T=1 (n=12, means $\pm$ SE) in comparison to the inlet water quality. Due to lack of significant differences between diets data have been pooled for diet. Organic wastes were removed at Day 1, and accumulated over the 5 day cleaning cycle. The sampling protocol is described in Figure 7.2. Data at T=0 have not been statistically compared with T=1; the change in water quality during the interruption in water exchange is presented and analysed in Figure 7.4.

The abscissa for DO and pH represent the influent water; TA in the influent water was not detectable. Inlet DO, TA & pH were determined at each sampling time by sampling the influent water into one of the experimental tanks.

Average temperatures at T=0/T=1 were as follows : Day 1/Day – 16.2/16.21°C; Day 1/Night – 15.9/15.1°C; Day 5/Day – 17.4/18.41°C; Day 5/Night – 16.8/15.81°C.

Different superscripts indicate significantly different means ( $P < 0.083$ ). As described in the main text, using a repeated measures ANOVA required calculation of an adjusted P value using the Bonferroni method.

Icons at the bottom of the figure indicate what is most likely to be having an impact at the indicated sampling time.



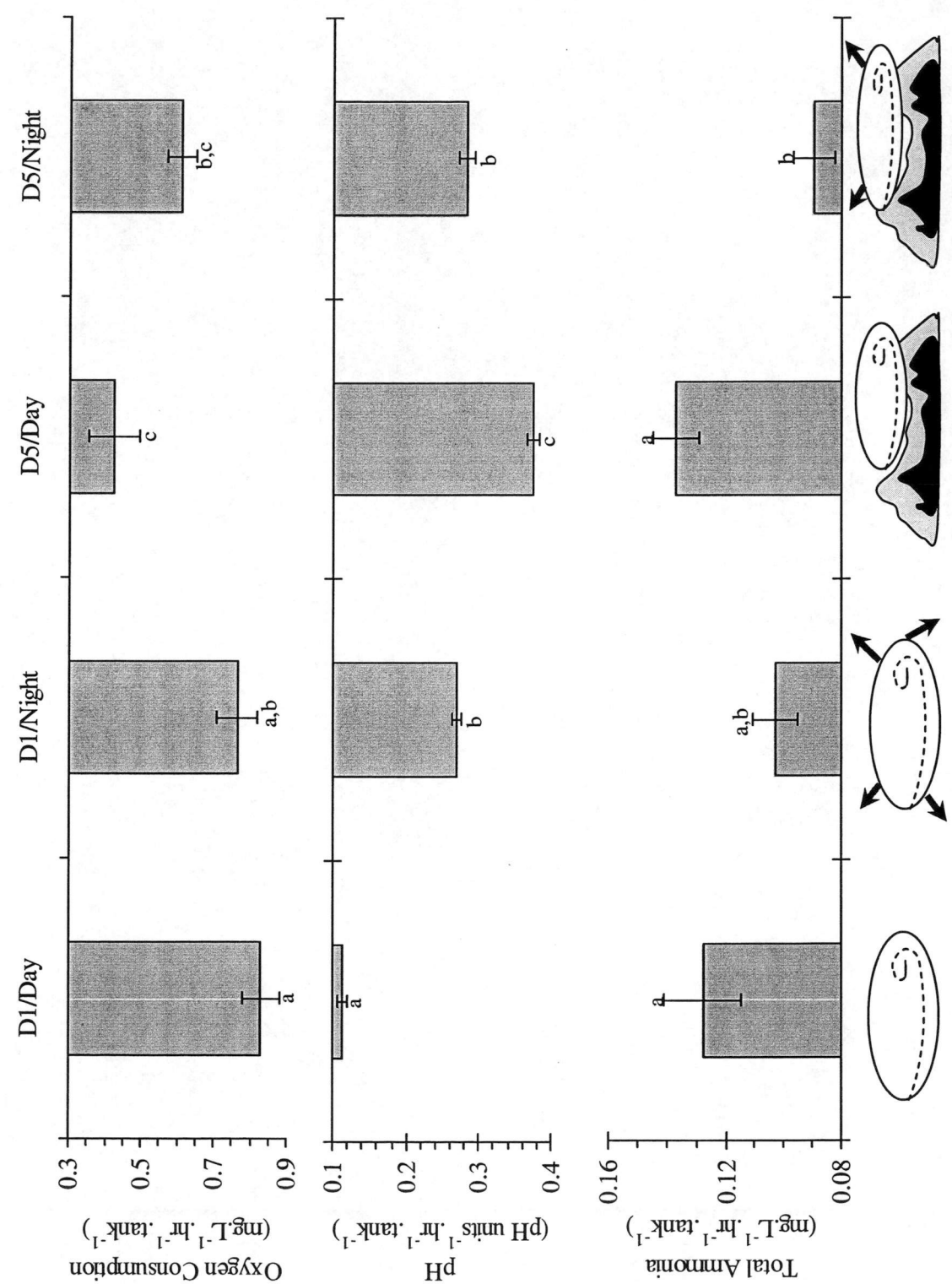


Figure 7.4 Rates of changes in water quality following an interruption in water exchange (T=1 in Figure 7.2) (whole tank metabolism) (means $\pm$ SE, n=12). Values above the abscissa indicate production and values below the abscissa indicate consumption. Different superscripts indicate significantly different means (P<0.083). As described in the main text, using a repeated measures ANOVA required calculation of an adjusted P value using the Bonferroni method. Icons at the bottom of the figure indicate what is influencing whole tank metabolism at the indicated sampling time, and are explained in Figure 7.3.

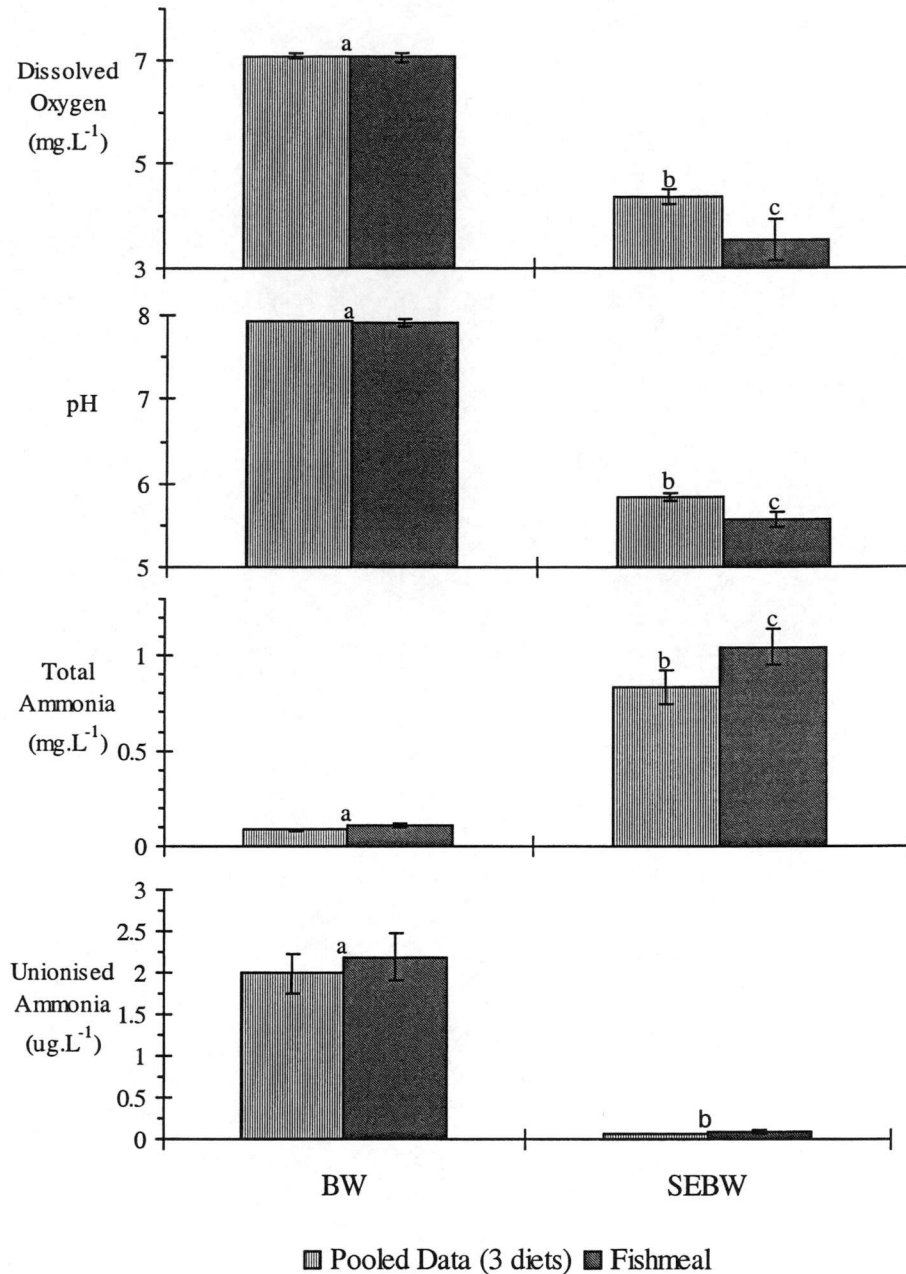


Figure 7.5 Comparison of BWQ and SEBW on Day 6 (means $\pm$ SE, n=9 for pooled data, n=3 for fishmeal). Superscripts indicate significantly different means ( $P < 0.05$ ). Data for the control, kaolin and fibre diets were pooled due to a lack of significant difference between these diets. BWQ data shown above was taken on Day 6 along with sediment enriched bulkwater (SEBW) sampled as described in the text.

## CHAPTER 8

### Nitrogen And Oxygen Budgets For Experimental Abalone Systems

#### 8.1 Introduction

Modern intensive culture practices can yield up to  $400 \text{ kg biomass.m}^{-3}$  ( $>7000 \text{ kg.ha}^{-1}$ ), compared to  $<1 \text{ kg.m}^{-3}$  ( $<100 \text{ kg.ha}^{-1}$ ) for the extensive systems common in rural third world communities (Stickney 1994; Wedemeyer 1996). Formulated diets are used in these systems to supply the nutritional requirements of the species being cultured, representing a substantial organic input that is only partially converted to biomass. Oxygen consumption, uneaten food, faecal wastes, leached nutrients and metabolic excretions from the stock can degrade water quality within the facility and effluent water leaving the system. The portion of the diet provided which is either not consumed or excreted as soluble metabolites or undigested faecal wastes constitutes a potential load on the wider environment (Gowen & Bradbury 1987; Beveridge *et al.* 1991).

Ammonia is a by product of protein catabolism, and in most marine organisms is excreted directly to the environment via the gills or kidney (Bayne & Newell 1983). Microbial activity will convert the nitrogenous component of particulate wastes into nutrients such as ammonia and nitrate which are more readily available to algae. Nitrogenous nutrients are commonly considered limiting for algal growth in marine environments and are a major concern to regulators (Gowen & Bradbury 1987).

Water quality degradation within the facility gives rise to a potential for growth reductions or stock health being compromised (Poxton 1991; Wedemeyer 1996; Martin *et al.* 1998; Person-Le Ruyet *et al.* 1997). Oxygen is traditionally described as a limiting factor for growth (Brett 1979), and chronic exposure to oxygen levels below saturation are known to depress growth in a range of



cultured aquatic species including abalone (Harris *et al.* 1999a). Ammonia is of concern to aquaculturists because of its known toxicity to aquatic species including abalone (Chapter 3; Harris *et al.* 1998a; Basuyaux & Mathieu 1999). While Chapter 3 shows that abalone are more sensitive in terms of growth when chronically exposed to environmental ammonia at oxygen levels below saturation, it is clear from Chapter 2 that abalone are remarkably resilient when exposed acutely to environmental hypoxia.

In order to avoid such degradation in water quality and its biological consequences, both regulators and aquaculturists must have an understanding of what factors influence water quality, and the contribution of different components in the system to the overall environmental load of the facility. Development of nitrogen budgets allows both parties to determine where the nitrogen entering the system is being distributed, and thus develop the most effective methods for reducing the net load on the environment. Oxygen budgets are of more concern to the aquaculturist, and provide fundamental information on how the system as a whole functions to provide an optimal environment for the species being cultured. In conjunction with data on what oxygen levels are limiting for growth, knowledge of how oxygen levels fluctuate over time and what influences the extent of these changes provide the aquaculturist with information on when oxygen levels may become limiting and how best to manage the system to maintain the optimal environment for the species being cultured.

Diet manufacturers are increasingly looking to use plant-based protein sources to reduce reliance on more expensive animal protein sources such as fish meal (Allan *et al.* 2000; Britz 1996a; Fleming *et al.* 1996; Fleming *et al.* 1998; Shipton & Britz 2001; Thiessen *et al.* 2003). Plant-based ingredients tend to have higher levels of indigestible organic and inorganic material (cellulose and ash), especially less processed ingredients which can be cheaper. The use of such ingredients may influence the nitrogen budget by altering the amount or composition of the organic wastes (Roden 1997). While the effect of stocking density on abalone growth has been investigated by several authors, these studies have concentrated on growth effects rather than water quality and environmental loading.

Classical respirometry techniques have traditionally been used on a laboratory scale to measure the effect of oxygen consumption or metabolic excretions on water quality, these results then being extrapolated to commercial stocking densities. However, there is a growing trend to use more commercially relevant stocking densities (Forsberg 1996), or even commercial scale grow-out tanks (Bergheim *et al.* 1991) in order to make the results more directly applicable to commercial systems. This paper uses data collected from different trials in small scale culture systems to look at the impact of diet formulation and stocking density on nitrogen and oxygen budgets for abalone systems.

## 8.2 Methods

The two trials which generated the data used in this chapter are fully described in Chapter 7 (Trial 1) and Chapter 5 (Trial 2).

### 8.2.1 Nitrogen Budgets

#### *Trial 1 (September-November 1998)*

Essentially a trial was conducted to investigate temporal changes in water quality for an experimental abalone grow-out system, and the influence of diet composition (Figure 8.1, Table 8.1). The experimental tanks were circular fibreglass units (diameter = 70 cm., volume = 55 L), with triplicate tanks allocated to each treatment. The 30 tanks used in the trial were enclosed within a heavily shaded area to prevent the development of algal biofilms.

The trial was divided into two phases, which are shown in Figure 8.1. Phase 1 of this trial generated data based on water quality readings of the bulk water (BWQ) taken over a 6 day period, including ammonia production data (Figure 8.1 and data presented in Chapter 7). Table 8.1 shows the variables measured for this chapter; data on dissolved oxygen, pH and ammonia being given in Chapter 7. The control diet was a proprietary formulation based on Soya flour and semolina. The three experimental diets consisted of 85% control diet and 15% test ingredient. Rice hulls (certified as pesticide free) ground to a fine powder were used as a source of indigestible organic matter, technical grade kaolin was used as indigestible inorganic matter and Tasmanian fish meal (based on jack mackerel) was used as a source of digestible nitrogen.

A sample of the organic wastes which accumulated over the cleaning cycle was collected on Day 6 for analysis of carbon and nitrogen content. The wastes were collected by siphoning 80-90% of the accumulated material into a glass bottle, avoiding any fragments of uneaten food, and allowed to settle for 30-60 minutes. The supernatant was then decanted, and residue transferred to 50-100  $\mu\text{m}$  mesh to remove excess water. The remaining wastes were then frozen, and freeze-dried prior to analysis. Carbon and nitrogen content was determined using an elemental analyser; ash was determined as the material remaining after 24 hours at 550°C. Samples of the diets were prepared for analysis in the same manner. All samples were ground to a fine powder prior to analysis. At the end of the trial, 2-3 abalone from each tank were held in pouches for 3-4 days to purge the gut contents prior to being frozen and subsequently freeze-dried for analysis of soft tissue carbon and nitrogen content.

In developing the budget, it was assumed that the formulated diet was the only source of nitrogen input into the tanks. A ration of 1% of biomass per day was used in calculating the nitrogen input, based on weighed ration, and moisture content of the diet was assumed to be 10% (ration=10.8 g dry matter<sup>-1</sup>.day<sup>-1</sup>), based on previous experimental diets used at this facility with similar composition. Food consumption was not measured directly. In determining nitrogen assimilation, digestibility of ingested nitrogen was assumed to be 80% for all diets (unpublished data; Fleming *et al.* 1996). Nitrogen assimilation was calculated as the amount of nitrogen digested minus the amount released as ammonia for each diet. Data from Trial 2 was used to calculate the organic waste production (Table 8.2), with the nitrogen content of organic wastes calculated using the data in Table 8.2. "Other Losses" was determined as the difference between the Daily N input from the diet and the sum of the calculated outputs. All data was calculated on a kg biomass per day basis.

Individual values for each component of the budget were calculated for each replicate tank and averaged to provide means for each diet. Data from each replicate tank were used for the statistical analysis.

*Trial 2 (December 1997-May 1998)*

This trial was based on a growth trial investigating the interaction between stocking density and refuge provision in cultured greenlip abalone, at the same facility as for Trial 1. The experimental system was based on circular, fibreglass tanks holding 220 L with central outlets and aerated with three air-stones per tank. The system of 18 tanks was located outside, arranged in three rows of 6, with individual black plastic covers fitted to prevent the formation of algal biofilms. Covers were briefly removed for feeding, tank cleaning and daily observation. Influent oceanic sea-water was supplied to the system at ambient temperature via header tanks and a 130 µm disc-filter, which was back-flushed daily. Influent water flow was set at 1.8 L.min<sup>-1</sup> per 120 abalone, with submersible aquarium pumps attached to the side of each tank used to standardise the total internal water flow in each tank to 12 L.min<sup>-1</sup>.

Growth data are presented in Chapter 5, and was based on individual measurements of 120 tagged individuals per tank. The individual weight gain for each individual was averaged to provide a mean for each tank, and then multiplied by the stocking rate to provide a biomass gain per tank. While there were 9 experimental treatments, only results from the treatments with no refuges (120, 240 and 360 abalone per tank) were used to develop the nitrogen budget. All nine experimental treatments were used for the oxygen budget, with treatments pooled for density. Towards the end of the growth trial, organic wastes from these treatments were quantitatively collected by siphon. After being allowed to settle, the supernatant was decanted and the wastes frozen for subsequent dry matter analysis. Some samples were also analysed for nitrogen and carbon content using an elemental analyser.

Nitrogen budgets were only developed for treatments with no refuges. The data and assumptions used in developing the nitrogen budgets for Trial 2 are shown in Table 8.2. It was assumed that the formulated diet was the only nitrogen input, with total N input for the trial calculated as the product of daily ration, N content of the diet (4.44%) and length of the growth trial in days for each tank. Nitrogen outputs were calculated for assimilation into whole live, abalone (biomass), solid organic wastes and ammonia production. Other losses were calculated as the difference between dietary N input and sum of the calculated

outputs. These components were calculated for individual replicate tanks, and averaged to provide a mean for each density.

The availability of growth data for this trial allowed assimilation data to be calculated. Since the nitrogen content was determined using dried whole abalone, the results were adjusted to allow for moisture content of the soft tissue. It was assumed for the purpose of this budget that the shell had negligible protein content. Live abalone were assumed to be 48% dry matter, based on data that show the shell is 37% of the live weight, and is 100% dry matter, while the remaining soft tissue (63% of live weight) is 20% dry matter (unpublished data; King *et al.* 1996; Sales *et al.* 2003). Nitrogen assimilation was then calculated as the product of biomass gain as dry matter and whole abalone N content from Trial 1 (Table 8.1). Nitrogen lost in the organic wastes was calculated from organic waste production data (as g dry matter.kg<sup>-1</sup>.d<sup>-1</sup>) collected at the end of the growth trial, and nitrogen content of these wastes. Organic waste production was determined by siphoning all the organic wastes that had accumulated over a cleaning cycle into a 20 L drum, through a screen to separate uneaten food. The collected wastes were allowed to settle for 2-3 hours then decanting the supernatant. The wastes were then drained further on paper towel before being dried in aluminium trays at 80°C for 24-48 hours. This was repeated several times for each treatment over the course of the growth trial. Ammonia production for the trial was calculated using ammonia production data for the control diet from Trial 1 (converted to g NH<sub>3</sub>-N.kg biomass.d<sup>-1</sup>). Other losses and the means were calculated as for Trial 1.

### **8.2.2 Oxygen Budget**

The oxygen budgets in both trials were based on using the experimental tanks as community respirometers to continuously measure oxygen consumption over several days as organic wastes accumulated. For both trials, one replicate tank on each treatment was de-stocked to provide a control for biofilm respiration. The tank outlets for 6 tanks at a time were plumbed into the solenoids of an open circuit respirometer. For 8.3 minutes in each 60 minute cycle, the effluent water from each tank was directed past an oxygen probe. Influent seawater was monitored for the remaining 10 minutes in each 60 minute cycle to provide a

calibration reading. A logger connected to the probe recorded the millivolt readings from the probe as the average for each minute. The average of the last 2 minutes was used to calculate oxygen consumption as milligrams of oxygen per unit biomass per hour, using the relationship given in Low (1995), in conjunction with the average biomass calculated from the abalone weighed at the end of the trial and the average influent water flow for each tank (recorded twice daily). The hourly readings for each tank were averaged for consecutive 24 hour (0000 - 2300) periods.

### *Trial 1 (Phase 2)*

As shown in Figure 8.1 the tanks were cleaned on Day 1 and one replicate tank for each diet was destocked using a spatula to remove the abalone. The stocked tanks were fed on Day 1 & 3 as normal. On Day 5 of the respirometry, the remaining 2 stocked tanks were destocked, using a spatula as before, and the oxygen consumption of the tanks was monitored for a further 24 hours with accumulated organic wastes but no abalone.

While the system had four components (abalone, organic wastes, biofilm and bulk water), the de-stocked tank provided a control for oxygen consumption by the bio-film and bulk water, and thus the measured changes in oxygen consumption are due to a combination of abalone metabolism and accumulated organic wastes. Oxygen consumption due to organic wastes was calculated as the percentage increase in oxygen consumption over the cleaning cycle.

### *Trial 2*

One replicate tank for each treatment was destocked using benzocaine, then thoroughly hosed out with seawater to remove benzocaine residue before being refilled. The stocked replicate tank was cleaned as normal. The outlets of 6 tanks at a time (3 treatments, each with one stocked and one de-stocked tank) were plumbed into the solenoids of the open circuit respirometer and logger used in Trial 1, and oxygen consumption was calculated the same way. Final biomass was calculated using the average weight of the tagged abalone multiplied by the stocking rate in a given tank, corrected for any mortality which had been recorded throughout the trial. The stocked tanks were fed daily as

normal. Oxygen consumption was calculated and averaged as for Trial 1. Treatments were pooled for density in developing the oxygen budget ( $n=3$  for each density).

### 8.2.3 Statistical Analysis

Homogeneity of variance was tested by observation of residual plots generated by JMP 3.2 (SAS Institute). ANOVA was used to determine significant treatment effects, with differences between means compared using Tukeys HSD (SPSS). Data from the statistical analyses (F ratio, DF, P) are given in Table 8.3.

## 8.3 Results

### 8.3.1 Trial 1

#### *Composition of diets, abalone tissue and organic wastes*

Table 8.1 shows the ash, carbon and nitrogen contents of the diets, organic wastes and abalone tissue from Trial 1. The test diets differed from the control diet by the addition of 15% test ingredient to the control diet, which led to significant changes in nitrogen and carbon content. The kaolin and fibre diets were lower than the control diet in terms of nitrogen, but not significantly ( $P>0.05$ ), while the fish meal diet was significantly higher in nitrogen than the control diet (186.97, 3, 4,  $P<0.001$ ). The control diet had the lowest ash content, while the kaolin diet was the highest. The ash content of the fibre and fishmeal diet diets was lower than the kaolin diet, but still significantly higher than the control diet (5287, 3, 4,  $P<0.001$ ).

Composition of abalone tissue and organic wastes followed a similar pattern to the diet, with wastes and tissue from the kaolin and fibre diets being significantly lower in nitrogen level than the control diet (tissue - 33.749, 2, 3,  $P=0.009$ ; wastes – 119.425, 3, 8,  $P<0.001$ ), and organic wastes from the fish meal diet significantly higher in nitrogen than from the control diet ( $P<0.05$ ).

#### *Nitrogen budget*

The nitrogen budget determined for Trial 1 is shown in Table 8.1 and Figure 8.2. Assimilation of ingested nitrogen was the biggest loss of nitrogen in this system, with organic wastes accounting for only a small fraction of the overall loss.

### *Oxygen Budget*

Figure 8.4 shows the oxygen consumption on a per tank basis for consecutive 24 h periods, including the period with no abalone. Lack of significant differences between diets allowed the data to be pooled for diet ( $P>0.05$ ). The increase at Day 4 over oxygen consumption at Day 2 (32%) is similar to the oxygen consumption of the organic wastes alone (34%). On Day 6, the abalone consumed 68% of the oxygen, and it was assumed that the abalone were consuming 100% of the oxygen at Day 2. Night-time oxygen consumption was always higher than day-time oxygen consumption, but not significantly different.

### **8.3.2 Trial 2**

#### *Organic waste production*

The lowest stocking density produced significantly lower amounts of organic waste on a dry matter basis (2.094, 2, 3,  $P=0.050$ ) than the medium and high densities, which were also significantly higher in nitrogen and carbon (nitrogen – 13.613, 2, 3,  $P=0.031$ ; carbon – 13.814, 2, 3,  $P=0.031$ ) (Table 8.2). Medium and high densities produced similar amounts of organic wastes, with similar levels of nitrogen and carbon.

#### *Nitrogen budget*

Nitrogen budgets constructed for each density treatment are shown in Figure 8.4. These budgets are based on the assumption that digestibility is not affected by stocking density. Actual nitrogen assimilation data was determined using growth data from the trial, rather than using an assumed nitrogen digestibility as in Trial 1.

#### *Oxygen budget*

Oxygen budgets based on community respirometry are shown in Figure 8.5. At the end of the experiment, the greatest percentage increase in oxygen consumption due to organic wastes was found at the lowest stocking density (34%), with the increase for medium and high stocking densities slightly lower



(25 & 29 % respectively). The results overall are similar to those for Trial 1. As for Trial 1, nocturnal oxygen consumption was generally higher than for the day time periods, but not significantly different ( $P>0.05$ ).

## 8.4 Discussion

There is a growing body of literature on various aspects of abalone culture, but the environmental aspects of abalone culture have not received as much specific attention. While more work is necessary to produce a more definitive nitrogen budget, this study provides some data and fills a gap in the current literature. In developing the nitrogen budgets for Trial 1, digestibility of nitrogen was assumed to be 80%, based on literature values (Fleming *et al.* 1996, 1998; Vandeppeer *et al.* 2002a) and unpublished data generated by the author from similar diets. Food consumption was not measured directly for either trial but was assumed to be 100% of the ration provided, which was weighed. Literature values indicate a range of abalone species fed formulated diets consume 0.05-1% biomass.day<sup>-1</sup> (Fleming *et al.* 1996; Britz 1996a, b).

### 8.4.1 Nitrogen Budgets

The results from Trial 1 show that approximately 20% of the nitrogen supplied to the abalone was being excreted as ammonia, and 20% as organic wastes or undefined losses (Figure 8.2). Cho & Bureau (1997) report that 50-60% of the ingested nitrogen in salmonids can be excreted as ammonia, and it is likely that at least a portion of nitrogen is being excreted in forms other than ammonia (Barkai & Griffiths 1987; Peck *et al.* 1987). These non-ammonia excretions would contribute to the “other losses”. Despite having the highest nitrogen level, ammonia excretion was lowest on the fishmeal diet. The protein level of the fish meal diet (39%) is in excess of the protein levels generally recommended for formulated abalone diets (20-30%; Uki & Watanabe 1992; Fleming *et al.* 1996). The results shown in Table 8.1 suggest that the excess nitrogen was excreted as organic-N in faecal waste since ammonia production and tissue nitrogen levels were no higher than the other diets. This may be due to abalone having a limited ability to digest excess protein, and thus excess protein is excreted in faecal wastes. Given that algal diets are generally much

lower in protein than formulated diets, this is not surprising. In contrast, in teleost species, increasing levels of dietary protein result in increased rates of ammonia excretion (Kaushik & Cowey 1991).

Increasing the level of indigestible material in the diet, either organic (cellulose in the fibre diet) or inorganic (ash in the kaolin diet) decreased the nitrogen content of the abalone tissue and organic wastes. Studies on digestibility have shown that increasing the cellulose in abalone diets has little effect on nutrient digestibility (Vandepeer *et al.* 2002a; Monje & Viana 1998), and neither does kaolin at the inclusion level used in Trial 1 (Vandepeer *et al.*, 2002b).

For Trial 2, the biomass gain data collected from this trial allowed nitrogen assimilation to be calculated. 20-35% of the nitrogen supplied in the diet was assimilated into abalone tissue, which is similar to a range of species including salmonids (Table 8.4). Neori *et al.* (1998) report that 14% of the nitrogen in algal diets, which have a much lower nutrient density than formulated diets, fed to the ormer (*Haliotis tuberculata*) was incorporated into tissue. In developing a theoretical model based on available literature, Maguire (1998) also calculated that 18% of the nitrogen supplied in formulated diets to abalone on commercial farms would be assimilated into tissue. While it would be expected that at the protein level of the fish meal diet, more protein would be assimilated into tissue, based on the response of *Haliotis midae* to increasing dietary protein (Sales *et al.* 2003) Loss of samples due to freezer failure prevented this hypothesis being tested.

Both trials were conducted at temperatures conducive to good growth rates in greenlip abalone (Gilroy & Edwards 1998), and therefore ammonia excretion rates would be close to maximum as well. Ammonia excretion rates in abalone have been shown to increase in summer and decrease during winter (McBride *et al.* 2001), which is the result of an increase in foraging activity in summer compared to winter (Donovan & Carefoot 1998).

The percentage of nitrogen not accounted for in nitrogen budgets calculated by other authors ranges from 8-55% (Krom *et al.* 1985b; Daniels & Boyd 1989; Hopkins *et al.* 1993). In the studies presented here, losses of nitrogen not accounted for ('other losses' in Figures 8.2 & 8.3) comprised 20-30% of the nitrogen added to the system. Some of these losses would be due to leaching of

soluble nitrogen compounds from the diet and organic wastes, excretion of non-ammonia nitrogenous metabolites such as urea, and other nitrogenous excretions such as mucous. Abalone generate fine particulate material due to the rasping action of the radula on the diet, an unknown portion of which is thus lost in the effluent water (Neori *et al.* 1998). Harris (1999) reported that, for abalone diets used in nutritional history trials, 7.4% of the total protein was water soluble. While not substantial, this would still make a contribution to the soluble nitrogen losses. In a study of diets for tropical abalone Jackson *et al.* (2001) also showed that protein leaches from immersed abalone diets. Mucous production is also likely to be a portion of the other losses (Barkai & Griffiths 1987; Peck *et al.* 1987; McBride *et al.* 2001). Mucous production in the ormer was shown to not differ significantly between summer and winter periods in the study of McBride *et al.* 2001.

Pore-water in the organic wastes has been shown to contain high levels of ammonia (Chapter 7) and potentially non-ammonia nitrogen as well. This would not be included in the organic waste component shown in Figures 8.2 & 8.3, which were based on a dry matter analysis of drained wastes. Nitrogen losses may also occur to atmosphere, and have been estimated at 10-30% in pond systems (Martin *et al.* 1998). While not likely to be important in either of the trials examined here, it may become more substantial in large scale abalone grow out tanks, which have a large air/water interface. Commercial facilities will also have an extensive system of drains, which if left open to the atmosphere, will also allow for ammonia to exit the system. Algal growth in these canals will also consume some of the nutrients released from the tanks. It may also be more relevant to some shallow tank designs, which have been developed in Australia, which have much larger surface area to volume ratios than deeper tanks.

The net nitrogen load on the environment is likely to be primarily derived from ammonia and other soluble, non-assimilated losses, since substantial amounts of the solid organic waste can be retained on site by proper solids treatment, thus reducing the net environmental load (Bergheim & Åsgård 1996). Improvements in solids removal, either during cleaning or at harvest, have substantially reduced the impact of pond systems on the environment (Bergheim & Åsgård

1996). Briggs & Funge-Smith (1994) reported that 30% of the nitrogen in marine prawn (*Penaeus monodon*) ponds was lost through the sediment. Essentially the contribution of the different components to the overall budget will be controlled by factors which affect growth, such as temperature and gonad maturation (McBride *et al.* 2001; Bayne & Newell 1983). Apart from directly affecting the rate of growth and protein utilisation, a secondary effect of season is the level of activity, which declines in winter and increases in summer (Donovan & Carefoot 1998).

Growth rates were not determined for Trial 1, but results from other trials in this system with similar densities and at similar temperatures indicate that growth rates would be in the order of 80-100  $\mu\text{m.d}^{-1}$  (Chapter 2). Growth rates recorded from Trial 2 (Chapter 5) showed that growth from the low density treatment (120 abalone/tank) was close to commercial growth rates for Australian abalone (80-100  $\mu\text{m.d}^{-1}$ ) (Fleming *et al.* 1996), although growth rates were slower in the higher density treatments. While growth rates vary with season, and nitrogen metabolism is influenced by growth and gonad maturation, these budgets were constructed from data gathered during the summer, or at temperatures conducive to optimum growth rates. Thus, it is considered that these budgets would represent the period of rapid growth ie maximum ammonia excretion and organic waste production (Donovan & Carefoot 1998; McBride *et al.* 2001).

#### 8.4.2 Oxygen Budgets

Aquaculturists are interested in oxygen budgets because of the implications for stock health should oxygen saturation decline sufficiently. An oxygen-depleted environment can lead to growth reductions, and impose a stress which can lead to secondary infections or mortality. Chapter 5 showed that low dissolved oxygen levels are likely to be found in conjunction with elevated environmental ammonia levels, which Chapter 3 showed can lead to growth reductions. It is also known that elevated environmental ammonia has a greater impact on growth at oxygen levels less than saturation (Chapter 3). Effluents with low DO are likely to have negligible impact on the receiving waters if the out-fall is suitably located. Oxygen budgets allow for the development of cost efficient aeration strategies, including predicting the requirements for supplementary

aeration. The data developed from the investigations in this paper, in conjunction with other work in the same systems (Chapter 7), also provide some more fundamental information on what influences water quality in abalone culture systems.

Oxygen budgets developed using whole tank respirometry show that the organic wastes consume between 25-34% of the oxygen in the system at the end of the cleaning cycle, with the two trials being in close agreement. Sediment oxygen demand (SOD) can be 50-70% of the total oxygen demand of pond systems (Colman & Jacobson 1991; Suplee & Cotner 1996). While not directly comparable with oxygen consumption as measured in the present study, it does show that accumulated sediments can consume substantial amounts of oxygen. Nocturnal activity led to an increase in oxygen consumption compared to day-time values, although the difference was not significant in either trial (shown for Trial 2 in Figure 8.5). Thus, interruptions to water exchange at the end of a cleaning cycle or at night will lead to more rapid declines in oxygen levels than interruptions during the day or at the beginning of the cleaning cycle. Given the nocturnal activity of abalone (Donovan & Carefoot 1998; Nakamura & Archdale 2001), night-time increases in oxygen consumption due to foraging activity are not unexpected, and have also been reported for the loco (*Concholepas concholepas*) a nocturnally active gastropod (Navarro & Torrijos 1994), the ormer (*H. tuberculata*, Peck *et al.* 1987; McBride *et al.* 2001) and for *Haliotis diversicolor supertexta* (Jan *et al.* 1981). McBride *et al.* (2001) found that the effect on nocturnal oxygen consumption in the ormer due to foraging was higher in the summer than in winter, as the time spent actively foraging tends to increase in summer and decline in winter. Increases in nocturnal oxygen consumption were observed in the current study from both trials, but the effect was not significant. Both Donovan & Carefoot (1998) and Nakamura & Archdale (2001) report that not all abalone will be active for any given foraging period, and there are several levels of activity between fully quiescent and actively foraging (Donovan & Carefoot 1998). This may explain why the increase in nocturnal oxygen consumption is variable, and not always significant.

Based on bioassay data, the declines in oxygen due to accumulation of organic wastes or nocturnal activity shown in Figures 8.4 & 8.5 are not likely to affect growth in abalone (Harris et al., 1998a). If these fluctuations are combined with elevated environmental ammonia levels, this may have impact on growth or health status (Chapter 3). If the oxygen levels decline below 80%, this may indicate that either water exchange is inadequate, or an excessive amount of organic wastes have accumulated. In this situation, health status may be compromised and the stock will be pre-disposed toward a secondary/opportunistic infection.

Table 8.5 shows some projected declines in oxygen levels and increases in ammonia following an interruption in influent water flow for a model culture system, based on data from Trials 1 & 2. The decline in oxygen level will be more rapid at the end of the cleaning cycle, due to presence of organic wastes. If growth rates were higher, it would also be expected that ammonia excretion and oxygen consumption would also be higher, and water quality would be degraded faster than shown in this projection. It is known that abalone are remarkably tolerant of acute changes in water quality, provided there is no underlying stress and stock health is not otherwise compromised (Chapter 2), so losses from a single episode may be low if water flow can be restored within 8 hours in this model. However, repeated episodes or a prolonged period with reduced water flow may lead to a compromised health status, and mortality from secondary infections.

## 8.5 Conclusions

Nitrogen budgets developed from experimental grow-out systems show that abalone generate a similar net environmental nitrogen load, in terms of  $\text{g N.kg biomass}^{-1}.\text{day}^{-1}$ , to a range of cultured species. Known nitrogen losses were primarily ammonia, with solid organic wastes a small fraction of the total nitrogen loss. Oxygen budgets showed that organic wastes can consume up to 25-34% of the total oxygen in system over 4-7 day cleaning cycle. While nocturnal foraging activity did increase oxygen consumption of the system, the effect was not significant.

	Experimental Diet			
	Control	Kaolin	Fibre	Fishmeal
<i>Diet*</i>				
(% of dry matter basis)				
% Nitrogen	4.84±0.24 <sup>a</sup>	4.13±0.16 <sup>a</sup>	4.59±0.39 <sup>a</sup>	6.32±0.38 <sup>b</sup>
% Carbon	38.95±0.91 <sup>a</sup>	32.00±0.85 <sup>b</sup>	37.00±0.43 <sup>a</sup>	47.30±0.42 <sup>c</sup>
% Ash	6.03±0.13 <sup>a</sup>	20.14±0.02 <sup>b</sup>	7.93±0.10 <sup>c</sup>	7.47±0.06 <sup>c</sup>
<i>Abalone Tissue**</i>				
(% of dry matter basis)				
% Nitrogen	5.59±0.45 <sup>a</sup>	2.32±0.44 <sup>b</sup>	3.10±0.44 <sup>b</sup>	.
% Carbon	26.93±1.10 <sup>a</sup>	18.60±0.97 <sup>b</sup>	20.32±0.97 <sup>b</sup>	.
<i>Organic Waste**</i>				
(% of dry matter basis)				
% Nitrogen	3.59±0.18 <sup>a</sup>	2.41±0.03 <sup>b</sup>	2.40±0.05 <sup>b</sup>	4.87±0.10 <sup>c</sup>
% Carbon	28.12±1.46 <sup>a</sup>	21.12±1.13 <sup>b</sup>	25.95±0.69 <sup>a</sup>	29.32±0.79 <sup>a</sup>
% Ash	31.10±3.13 <sup>a</sup>	46.54±0.83 <sup>b</sup>	31.05±2.95 <sup>a</sup>	30.46±1.43 <sup>a</sup>
Ammonia Production (mg NH <sub>3</sub> .kg biomass <sup>-1</sup> .d <sup>-1</sup> )	4.37±0.30	3.98±0.36	4.80±0.44	3.99±0.46
Daily N Intake (g N.kg biomass <sup>-1</sup> .day <sup>-1</sup> )	0.44	0.37	0.41	0.57

Table 8.1 Composition of diets, abalone, organic wastes and effluent water for Trial 1, used to calculate the outputs shown in Figure 8.2. Other losses was determined as the difference between the sum of the calculated outputs and daily nitrogen intake. Assimilated nitrogen was determined by subtracting the nitrogen excreted as ammonia from the nitrogen digested (assuming nitrogen digestibility was 80%). It was assumed that all the ration provided (12 g fresh diet, 10.8 g dry matter.day<sup>-1</sup>) was consumed.

Different superscripts indicate significantly different means ( $P<0.05$ ) with in each row. Freezer failure resulted in loss of abalone samples from the fishmeal diet.

\*\* values are means  $\pm$  SE, n=3

	Abalone per tank		
	120	240	360
Daily ration (g.day <sup>-1</sup> )	22.25±1.63	47.26±1.21	72.35±0.64
Days of trial for replicate tanks	91, 69	115, 80	116, 95
Average biomass (kg)	3.57±0.01	6.67±0.09	10.30±0.22
Biomass gain (kg)	1.07±0.24	1.70±0.04	3.00±0.426
Total N input (g)	77.76±9.50	202.07±46.29	335.64±44.31
<i>Distribution of N</i>			
Assimilated into tissue (g N)	29.74±6.62	47.08±1.27	83.07±11.83
Ammonia Production (g N)	25.48±5.06	59.80±15.96	96.86±13.41
Organic wastes (g N)	8.78±1.74	28.66±7.65	48.85±6.76
Other losses (g N)	-1.12±7.24	42.99±20.74	65.31±6.38
<i>Composition of Organic Wastes</i> (% of dry matter)			
Nitrogen	1.92±0.61 <sup>a</sup>	2.67±0.25 <sup>b</sup>	2.81±0.13 <sup>b</sup>
Carbon	15.00±5.20 <sup>a</sup>	21.40±2.15 <sup>b</sup>	22.47±1.73 <sup>b</sup>
Organic Waste Production (g.d <sup>-1</sup> .kg biomass <sup>-1</sup> )	1.56±0.04 <sup>a</sup>	1.68±0.04 <sup>a,b</sup>	1.76±0.00 <sup>b</sup>
Food Conversion Ratio (kg dry ration provided.kg biomass gain <sup>-1</sup> )	1.50±0.15	2.43±0.49	2.29±0.02

Table 8.2 Data and assumptions used to determine nitrogen budgets for Trial 2. Unless specified, data are means for replicate tanks at each density (n=2, ±SD). A nitrogen content of 5.59% of dry matter for whole, dried abalone was used (control diet from Trial 1). The diet used was 4.44% nitrogen on a dry matter basis.



	Factor	n	F ratio	Df	P
<b><u>Trial 1</u></b>					
<i>Diet Composition</i>					
Carbon	Diet	3	18.481	3,4	0.008
Nitrogen	Diet	3	186.979	3,4	0.000
Ash	Diet	3	5287.756	3,4	0.000
<i>Tissue Composition</i>					
Carbon	Diet	2	25.464	2,3	0.013
Nitrogen	Diet	2	33.749	2,3	0.009
<i>Organic Waste Composition</i>					
Carbon	Diet	12	11.579	3,8	0.003
Nitrogen	Diet	12	119.425	3,8	0.000
Ash	Diet	12	11.539	3,8	0.003
<i>Ammonia Production</i>	Diet	12	1.293	3,8	0.342
<i>Oxygen Consumption</i>	Day	6	12.900	3,24	0.000
<b><u>Trial 2</u></b>					
<i>Organic Waste Production</i>	Density	2	9.024	2,3	0.050
<i>Organic Waste Composition</i>	Density				
Carbon			13.814	2,3	0.031
Nitrogen			13.613	2,3	0.031
<i>Oxygen Consumption</i>					
120 abalone/tank	Day	3	3.461	5,12	0.036
240 abalone/tank	Day	3	4.854	5,12	0.012
360 abalone/tank	Day	3	5.536	5,12	0.007

Table 8.3 Results from statistical analysis of diets, organic wastes and abalone tissue ammonia production and oxygen consumption presented in Tables 8.1 & 8.2, and Figures 8.2-8.5.

Source	Species	% Nitrogen Assimilation	Nitrogen Loading
			$\text{g N. Kg biomass}^{-1}.\text{d}^{-1}$
Trial 2	greenlip abalone	20-35	0.15 – 0.23
Bergheim & Åsgård 1996	Atlantic salmon, Norway	50	
	Channel catfish	25	
Briggs & Funge-Smith 1994	<i>Penaeus monodon</i> , Thailand	20-25	
Brune & Eversole 1993	Crawfish ponds, South Carolina	23	
Neori & Krom 1991	<i>Sparus aurata</i> Tanks and ponds	26-27	
Cripps & Kelly 1996	Various salmonid systems		0.21 - 0.29
Axler <i>et al.</i> 1997	rainbow trout, commercial raceways		0.16 – 0.36

Table 8.4 Literature values for nitrogen assimilation and loading, compared with results from Trial 2

	Oxygen Saturation	Total Ammonia
<b><i>Following an Interruption to Water flow</i></b>		
After 1 hour Day 1	76%	0.09 mg.L <sup>-1</sup>
After 1 hour Day 5	71%	
After 4 hour Day 1	44%	0.35 mg.L <sup>-1</sup>
After 4 hour Day 5	23%	
<b><i>Time to 0% Oxygen Saturation</i></b>		
Day 1	8 hour	0.69 mg.L <sup>-1</sup>
Day 5	5 hr 30 min	

Table 8.5 Projected declines in oxygen level from an interruption in water exchange. The projection is based on a 10 000 l tank holding 200 kg of abalone, with an influent water flow of 200 L.min<sup>-1</sup>, at 18°C and 100% oxygen saturation (7.5 mg.L<sup>-1</sup>). Based on data from the current study, oxygen consumption is assumed to be 40 & 60 mg.kg biomass<sup>-1</sup>.hour<sup>-1</sup>. Ammonia production is taken from the control diet in Trial 1 (4.37 mg.L<sup>-1</sup>).

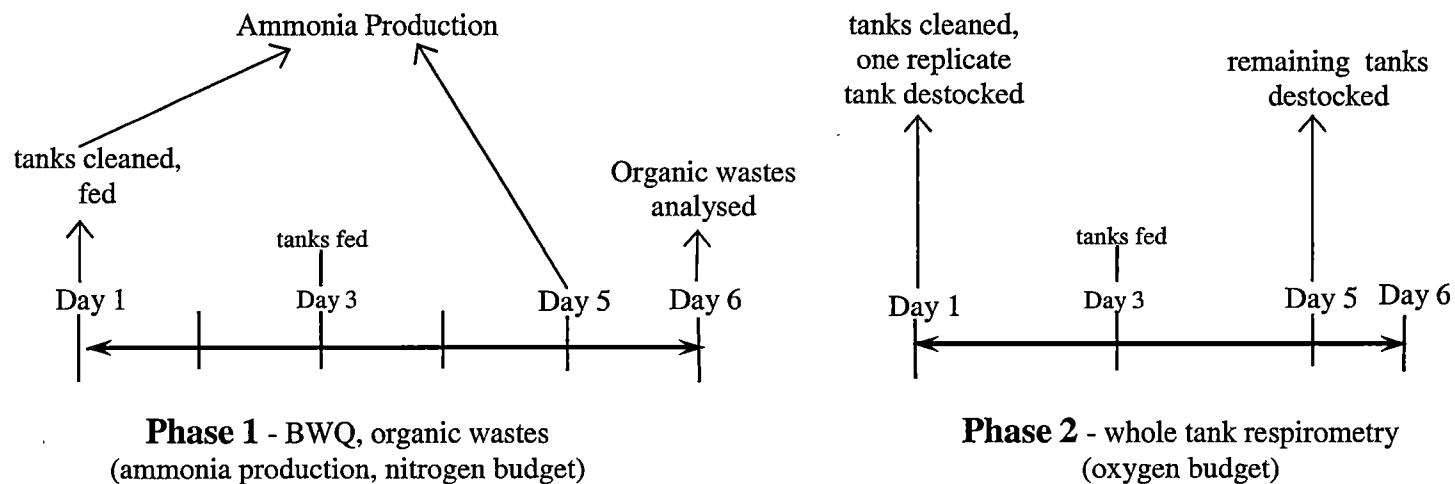


Figure 8.1 Experimental schedule for Phase 1 & 2 in Trial 1. In Phase 1, ammonia production was determined at night and during the day as indicated, and a sample of organic wastes was collected on Day 6 for analysis of C & N (on a dry matter basis). Ammonia production was higher during the day than at night, but did not differ between Day 1 and Day 5. Daytime ammonia production figures were used in calculating the nitrogen budgets. In Phase 2, the tanks were used as community respirometers for measuring oxygen consumption, as described in the text. One tank was destocked on Day 1 to provide a control for biofilm respiration. The remaining tanks were destocked on Day 5, leaving the accumulated organic wastes behind, and oxygen consumption monitored for a further 24 hours.

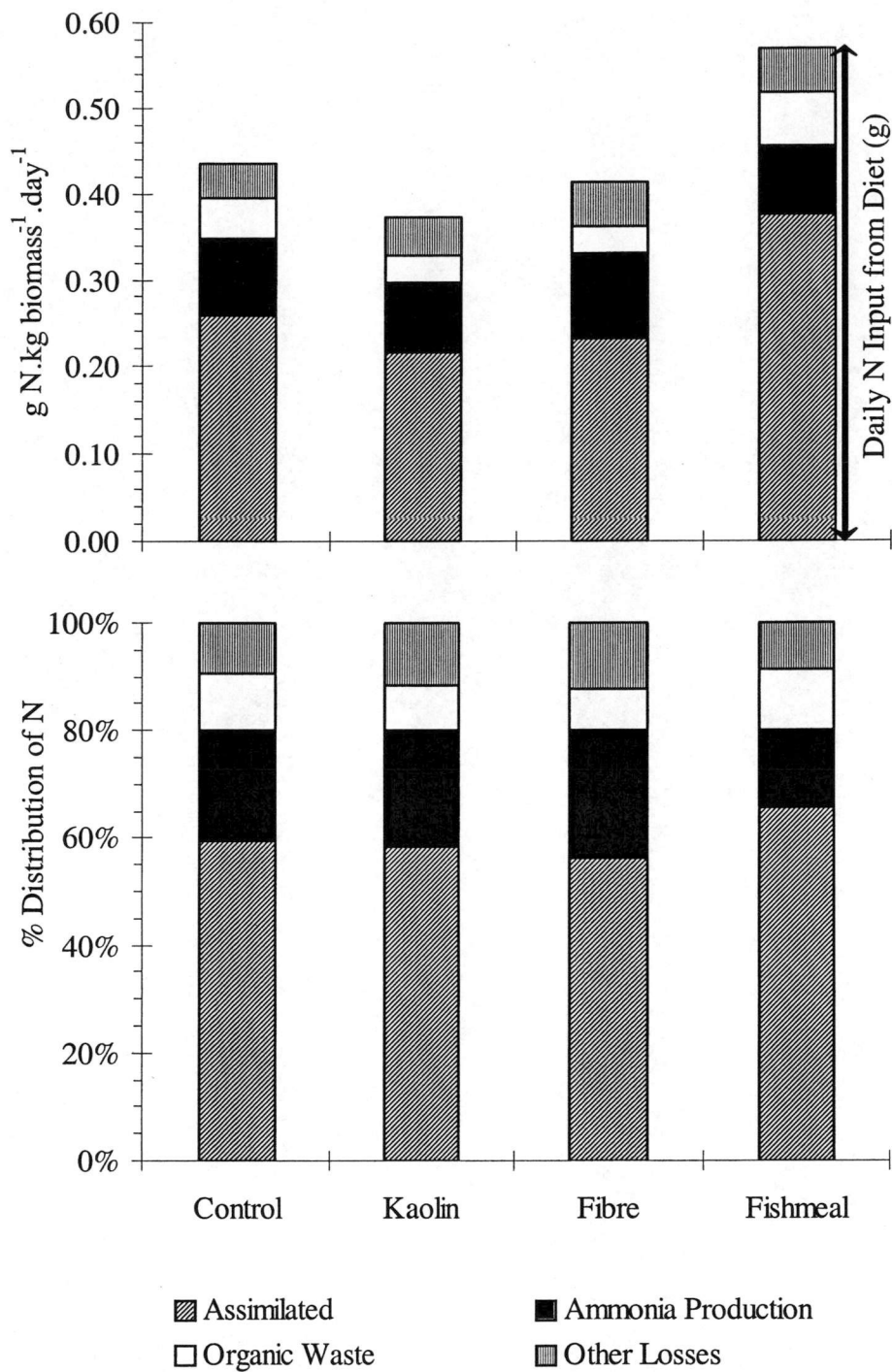


Figure 8.2 Nitrogen budget from Trial 1. The calculations are fully explained in the text. Test ingredients were 30% of the control diet (air-dried basis) (Table 8.1). Calculation of the known outputs is described in the text, and “Other Losses” was determined by subtracting the sum of the known nitrogen outputs from the daily nitrogen input. Calculation of daily N input the product of dry matter intake ( $10.8 \text{ g.tank}^{-1}/\text{day}^{-1}$ ) and the nitrogen content of the diet (Table 8.1).

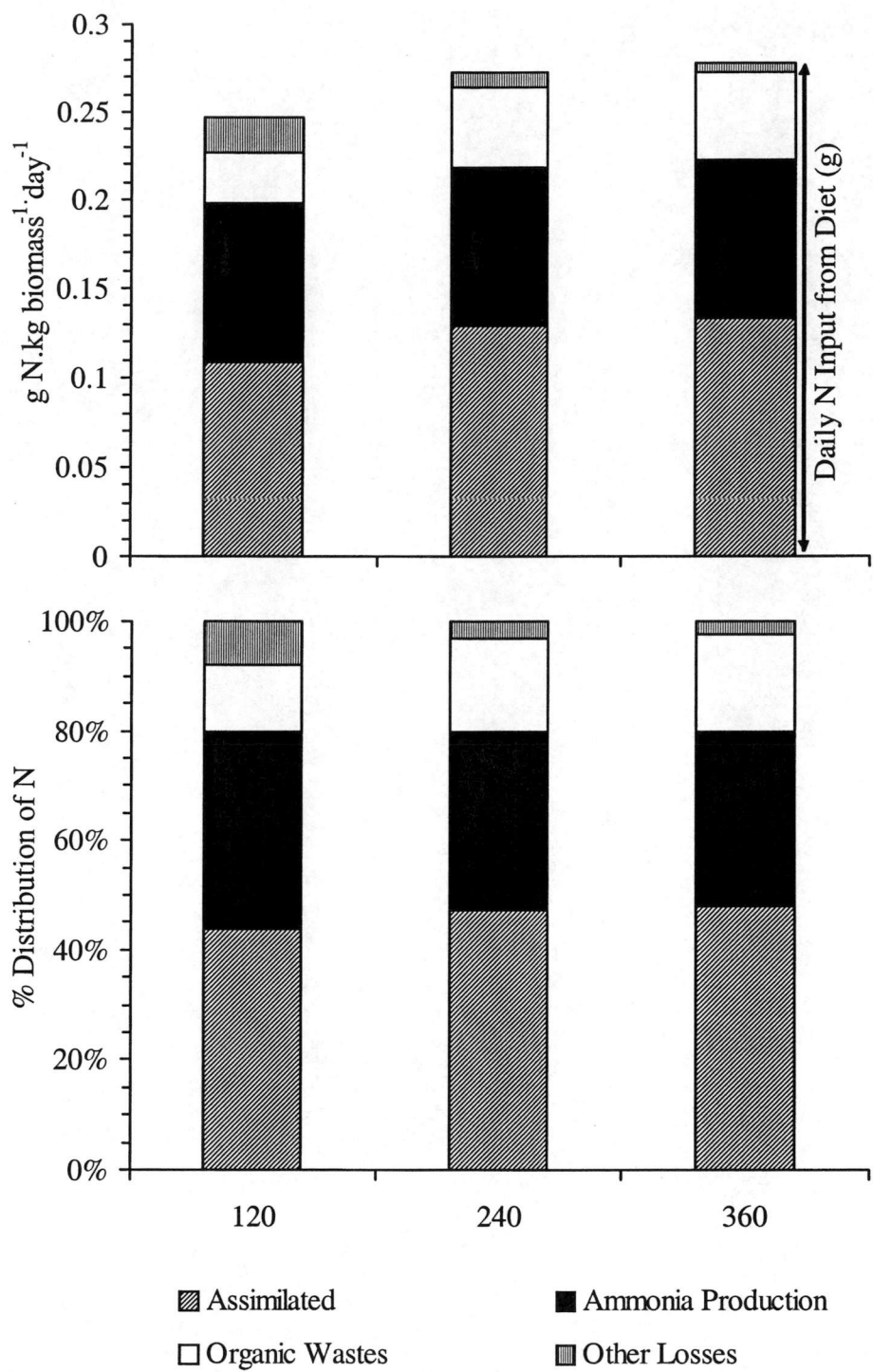


Figure 8.3 Nitrogen budget for Trial 2. The inputs were calculated as g N.(average kg biomass for trial)<sup>-1</sup>.day<sup>-1</sup>. Other losses were determined as the difference between the sum of the calculated outputs and the daily nitrogen input from the diet.

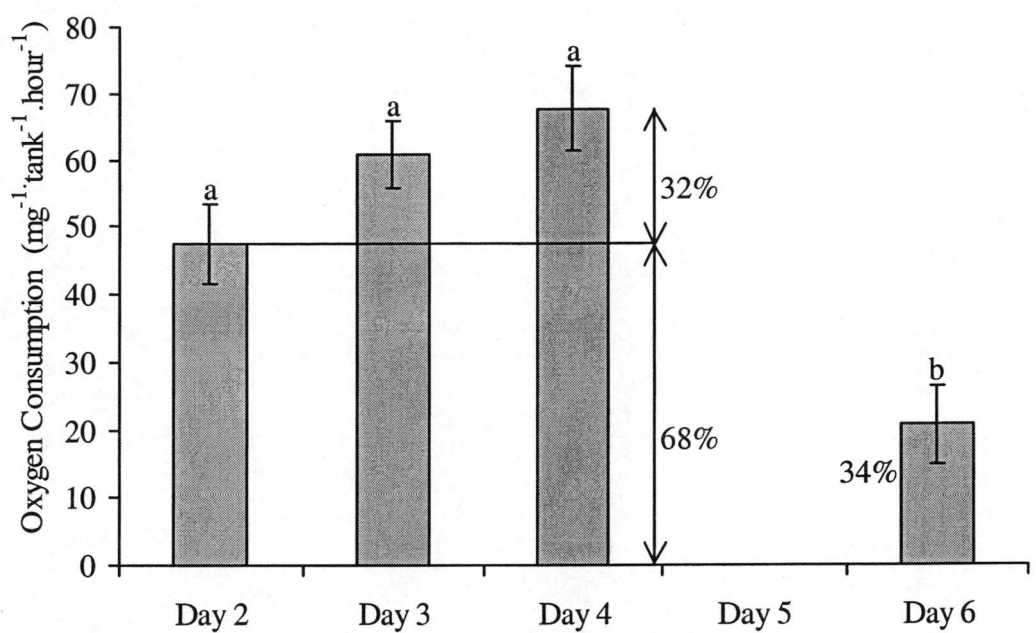


Figure 8.4 Oxygen Budget from Trial 1 (means±SE, n=7) for whole 24 h periods (00.00-23.00) showing percentage consumption of wastes and abalone at Day 4. The tanks were destocked at Day 5, and the value for Day 6 is oxygen consumption by wastes alone. Tanks were cleaned on Day 1, and Day 2 is assumed to be free of organic wastes. Average biomass in each tank was 1.2 kg. Destocking the tanks precludes expressing the consumption per unit biomass.

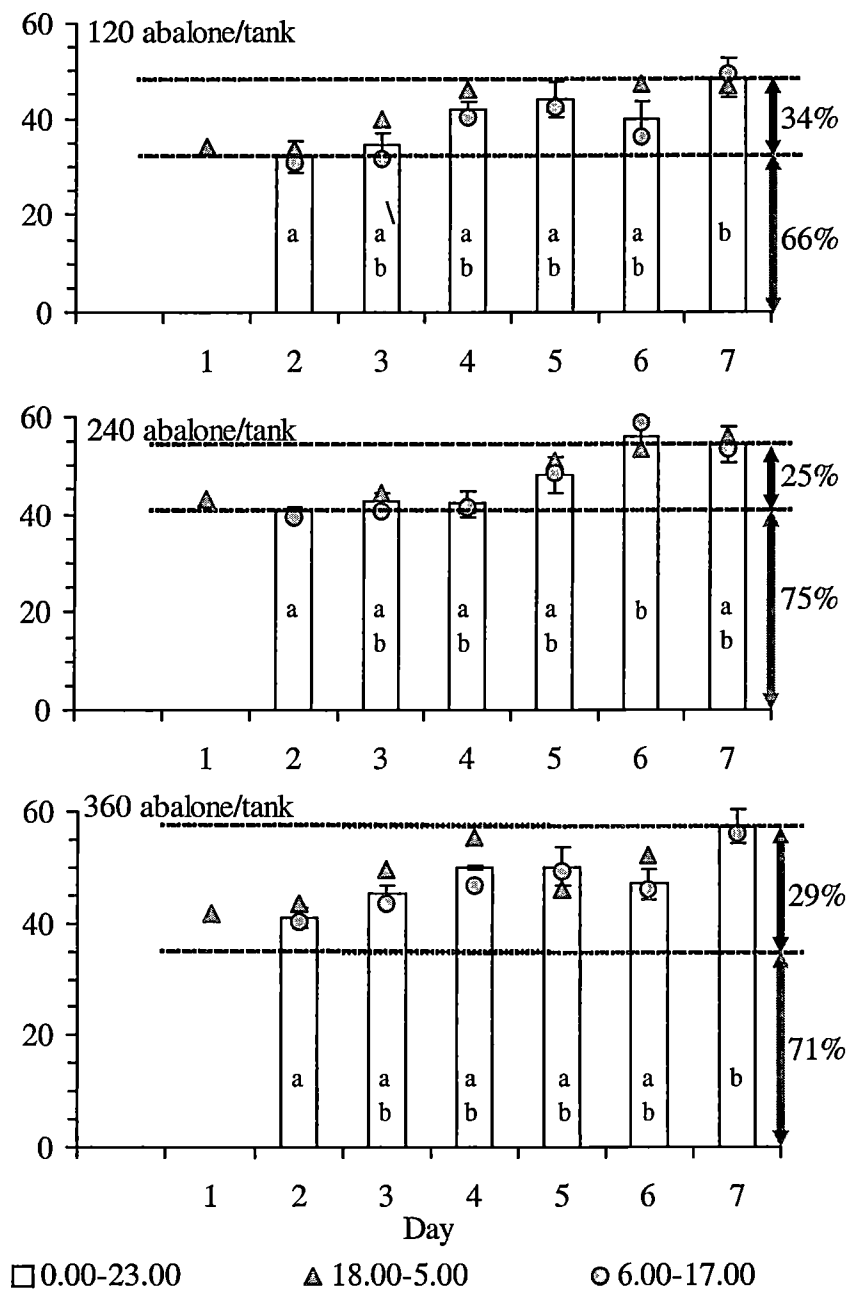


Figure 8.5 Oxygen budget for Trial 2 (means  $\pm$  SE,  $n=3$ ). Arrows and figures indicate percentage consumption by organic wastes and abalone at Day 7. Tanks were cleaned on Day 1, and thus Day 2 is assumed to be free of organic wastes. Different letters indicate significantly different means with-in each density ( $P<0.05$ ). Asterisk indicates significantly different means between densities ( $P<0.05$ ).



## Chapter 9

### General Discussion

The broad scope of this study covers a range of husbandry and system management issues influencing growth and water quality in abalone culture systems. It addresses the effect on growth of both intermittent (Chapter 2) or chronic (Chapter 3) exposure to low dissolved oxygen levels combined with elevated ammonia concentrations. Using serum ion data, some fundamental physiological effects of environmental ammonia exposure were also investigated (Chapter 4). While increasing stocking density did reduce growth rates, Chapter 5 showed that this could be mitigated by increasing the level of refuge provision. This would be due at least in part to a reduction in the effort required for foraging and finding a refuge (Chapter 6). It is generally accepted that elevated ammonia and low dissolved oxygen levels are the water quality variables most likely to impact on growth and health status of cultured stock in modern high density systems, and the water quality data from the refuge/density trial (Chapter 5) and Chapter 7 indicate that this will hold true for abalone culture. Environmental loadings in terms of nitrogen and oxygen were also determined for pilot scale growout systems (Chapter 8).

Table 9.1 shows the variables in the present study that have an impact on growth, while Figure 9.1 summarizes the overall findings in the context of a generalized abalone growout system.

## 9.1 Impacts of Elevated Environmental Ammonia Combined with Low Dissolved

### Oxygen Levels

Published growth data shows that greenlip abalone are relatively sensitive to elevated environmental ammonia concentrations and low dissolved oxygen levels as individual stressors (Table 9.2). There is also evidence from Chapter 3 that modest declines in dissolved oxygen level (80% saturation) combined with low environmental ammonia concentrations have a greater impact on growth than as individual variables. However, growth rates were actually higher at low dissolved oxygen levels (60% saturation) combined with the same levels of ammonia. In comparing the results of Chapter 2 (Intermittent Exposure) and Chapter 3 (Chronic Exposure), it is evident that while abalone growth can be significantly reduced by chronic exposure to low DO and elevated ammonia levels, healthy abalone are remarkably tolerant of short term and intermittent exposure to high levels of ammonia and low dissolved oxygen levels.

The capacity of many molluscs, including abalone, to survive hypoxic episodes is well established (eg. Brix *et al.* 1979; Ainslie 1980; Brix 1983; McMahon 1988; Wells *et al.* 1998a). If the biology and eco-physiology of haliotids are considered, this is not surprising. Abalone are known to aggregate in the wild during the daylight quiescent period, commonly under or around rocks, or in crevices (Poore 1972; Shepherd 1973; Shepherd & Partington 1995). While the shell is designed to induce water movement through the gill cavity (Voltzow 1983; Tissot 1992), it is unlikely that this will be able to fully supply the oxygen requirements during the inactive quiescent period. Oxygen levels around these aggregations could also be expected to decline at times of low water exchange and warm weather. The pedal muscle would also require substantial amounts of oxygen during either sustained activity such as foraging or intense periods of activity such as escaping from predators or righting the inverted animal. Since the pedal muscle can be up to 60% of the live weight of an abalone (Jorgensen *et al.* 1984), it is generally considered that the relatively simple circulatory system abalone have

would be unable to supply sufficient oxygen for fully aerobic function (Gäde 1988; Wells *et al.* 1998a). Coupled with the fact that the pedal muscle is known to receive a smaller proportion of the blood flow than other organs (Jorgensen *et al.* 1984), and that blood flow is restricted to the pedal muscle during exercise (Russell & Evans 1989), it is clear that a functional hypoxia will rapidly develop, requiring this muscle to function anaerobically.

Better growth rates at low (60% oxygen saturation) compared to medium (80% oxygen saturation) DO levels were not expected. It is possible that at 80% saturation the abalone have not fully activated the response to hypoxia, and are attempting to function aerobically in an oxygen limited environment, at a substantial energetic cost. At 60% saturation, it is possible that the abalone are able to fully activate the biochemical response to hypoxia which is more energy efficient in the hypoxic environment, and more energy is available for growth. Heart rate in abalone declines with ambient oxygen concentration (Nimura & Yamakawa 1989; Russell & Evans 1989), and therefore uptake of water borne toxicants such as ammonia may be reduced at lower oxygen levels. It is therefore possible that the low oxygen levels in either the pulse exposure (Chapter 2) or chronic exposure (Chapter 3) may have reduced exposure of the internal tissues to ammonia, which may explain the improvement in growth at low oxygen levels seen in Chapter 3, compared to growth at medium oxygen levels. Abalone in the LO treatments may also have a reduced metabolic rate compared to abalone at the medium oxygen level (Harris *et al.* 1999a), which may have further mitigated the biochemical impact of ammonia.

Phospho-arginine would probably be used to generate ATP during the initial stages of hypoxia, followed by the fermentation of glycogen and aspartate (Gäde 1983; Gäde & Ellington 1983; Hochachka *et al.* 1983; Wells *et al.* 1998a). In abalone, the supply of NAD<sup>+</sup> for glycolysis is maintained by the formation of reductive condensation of pyruvate with taurine, which produces tauropine (Gäde 1988; Ryder *et al.* 1994; Wells & Baldwin 1995; Baldwin *et al.* 1992). D-lactate is also produced during anaerobic glycolysis (Ryder *et al.* 1994).

The capacity to function during hypoxia is finite and anaerobic mechanisms are not as efficient at producing ATP, and thus chronic exposure as in Chapter 3 or Harris *et al.* (1999a), does result in growth depression. Gäde (1988) also demonstrated the finite capacity of abalone to cope with hypoxia, with mortality resulting after 8 h of anoxia in *Haliotis lamellosa*. This is further highlighted by the results of the hypoxic event in Chapter 3, which occurred due a lack of influent water into the bioassay units for 20 hours. Blacklip abalone demonstrated a greater tolerance to hypoxia, based on the limited mortality observed for this species following the same event. This is a likely consequence of the fact that blacklip abalone are commonly found much higher up the shore line than greenlip abalone (Shepherd 1973), frequently in the inter-tidal zone, and would have a greater chance of aerial exposure or experiencing low DO levels than greenlip abalone. Similar relationships between the ability to withstand severe environmental conditions and predominantly inter-tidal habitat have been demonstrated for a range of molluscan and crustacean species (Taylor & Spicer 1987; Morton 1990).

The potential for pre-exposure to environmental ammonia to improve survival to more severe conditions is suggested by the pattern of mortality following the hypoxic event in Chapter 3. While there was substantial mortality in treatments at low and medium ammonia levels (1.06 & 1.88 mg TA. L<sup>-1</sup>), mortality was much lower in abalone previously exposed to high levels of ammonia (8.07 mg TA. L<sup>-1</sup>). It is possible that this exposure, which also resulted in the lowest growth rates, had such an impact on metabolism that the hypoxic episode had less of an effect than it would on healthy abalone.

Environmental ammonia exposure in both vertebrate and invertebrate species has been shown to disturb ion exchange and serum chemistry. Chapter 4 also shows that ammonia exposure in abalone also disrupts normal ion regulation and serum chemistry. Osmoregulation in abalone is controlled by organic osmolytes rather than inorganic ions, so any disturbance in osmoregulation is difficult to determine from this study. However, ammonia is known to interfere with glycolysis in both vertebrate and invertebrate species, which would have a direct impact on ATP

production. Given that ion regulation is inherently energy intensive, any disruption in these processes will increase the requirement for ATP. This decrease in ATP production coupled with an increased demand for ATP for ion regulation may explain the growth reductions observed in Chapter 3. This would be further exacerbated by the fact that while abalone can use other pathways for ATP production during hypoxic episodes, the yield is lower than for aerobic glycolysis.

## 9.2 Stocking Density, Refuge Provision & Abalone Behaviour

The juvenile stages of the abalone species cultured in Australia are well known to be cryptic during the daytime resting period, with wild stocks taking refuge in the crevices of rock formations or between boulders (Shepherd 1973; Shepherd & Partington 1995). The density of natural aggregations is at least partly controlled by the available refuge area (Shepherd & Partington 1995). It is not surprising that more abalone used refuges as they became available (Chapter 6). As with studies on wild populations of herbivorous gastropods and cultured abalone, an increase in stocking density led to a decrease in growth (Chapter 5). It is likely that this is partly due to the increase in crowding and stacking as the number of abalone per tank increases making it more difficult to forage effectively and increasing the energy required for locomotion. The cost of locomotion in gastropods is higher than other genera due to the requirement for mucous, which can be up to 30% of the energy budget (Denny 1980; Peck *et al.* 1987; Donovan & Carefoot 1998).

Increasing the level of refuge provision at medium and high densities improved growth rates in terms of both length and weight. Chapter 6 showed that as the number of refuges per tank increased, there were fewer abalone per refuge. This would have made it much easier for abalone to emerge from the quiescent period and commence foraging. There is also considerable evidence that not all abalone will actively forage on a given night, with a number of different activity levels between fully active and fully quiescent observed in studies on different species (Donovan & Carefoot 1998; Nakamura & Archdale 2001). Increasing the number of refuges would reduce the disturbance of these abalone by those emerging

to forage, which may be important physiologically. Emergent abalone would also find it easier to emerge if the refuges were less crowded. As the refuges also decreased the effective foraging area (ie flat floor area of the tank where food would be available for grazing), it would be expected that food abundance would have been increased, which in turn would improve foraging efficiency. The presence of refuges would also make it easier to find a suitable resting area for the daytime quiescent period.

From the above discussion, it can be seen that provision of refuges, and increasing the level of refuge provision, has the potential to substantially reduce the energy requirement for foraging. This potentially allows more energy to be available for somatic growth, assuming no other factor is diverting energy from growth such as gonad maturation.

Water quality measurements taken under the refuges did not detect any substantial differences from the bulk water, even at 360 abalone/tank despite the accumulation of organic wastes under the refuges over the cleaning cycle. This may be a consequence of the fact that the circular water flow generated in the circular tanks used in this trial maintained a degree of water exchange under the refuges, even though it was very low at the highest density with 12 refuges/tank (based on observations with food dye). Without this circular water current, it is likely that water exchange under the refuges would have been reduced or absent, and Chapter 7 suggests that reductions in water quality (increased ammonia, decreased DO, pH) from a combination of abalone metabolism and accumulating organic wastes would be expected.

Influent water flow was set at  $1.8 \text{ L} \cdot \text{min}^{-1}$  per 120 abalone in order to prevent water quality become a confounding factor. A difference in dissolved oxygen and total ammonia levels was still evident between low and high stocking densities, although the effect of the changes would be unlikely to impact on growth. This highlights the role of water exchange in maintaining water quality, and setting influent water flow at an appropriate level for the biomass in the system.

### 9.3 Oxygen Budgets & Factors affecting Water Quality

As stocking density increased in Chapter 5 (stocking density/refuge provision), TA concentrations increased and DO levels decreased, although setting water exchange on a per animal basis prevented conditions developing that were likely to affect growth, based on the results from Chapter 3 (chronic exposure). In Chapter 7 (temporal effects), dissolved oxygen levels declined both at night during nocturnal foraging activity, and over several days as organic wastes accumulated. Chapter 8 (budgets) showed that, assuming 100% of the oxygen in the system is being consumed by the abalone at the start of the cleaning cycle, only 68-75% of the oxygen is consumed by the abalone after 5 days, with up to 32% being consumed by accumulated organic wastes. This would account for the significantly lower oxygen levels in the bulk water at Day 5 in the cleaning cycle compared to Day 1 in Chapter 7.

Nocturnal oxygen consumption increased but not significantly due to foraging activity when measured by whole tank respirometry in Chapter 8 (budgets), and on Day 5 in Chapter 7. Community metabolism studies in Chapter 7 (temporal effects) also showed no difference in oxygen consumption at Day 1 between day and night, but did show significantly higher oxygen consumption at Day 5/Night than during Day 5/Day. Bulk water readings on Day 1 in Chapter 7 also reveal significant declines in dissolved oxygen level at night compared to daytime readings. Nocturnal increases in oxygen consumption by abalone have been reported elsewhere (Jan *et al.* 1981; Peck *et al.* 1987). The greater increase in oxygen consumption detected at Day 5/Night in the community metabolism studies may also be partly due to the abalone disturbing oxygen-depleted wastes, which then consumed oxygen from the water column.

Ammonia production was found to be significantly higher during the day than at night (Chapter 7 (temporal effects)), although normal water exchange prevents ammonia accumulating to deleterious levels. Such a short term increase in ammonia production probably results from catabolism of ingested protein and other biochemical processes. Over longer period (5 days), ammonia levels in the bulk

water actually declined, which may be due microbial processes in the wastes consuming ammonia. Given that the rate of ammonia production did not change over the cleaning cycle, the observed decline in ammonia level in the bulk water is unlikely to be the result of some longer term temporal pattern of ammonia excretion by the abalone.

Water flow in commercial systems needs to allow for these alterations in water quality, rather than being based on oxygen consumption only at Day 1 of the cleaning cycle.

Once water exchange is interrupted, Chapter 7 (temporal effects) shows that water quality changes rapidly in terms of pH, DO (both declining) and TA (increasing). In Chapter 2 (intermittent exposure), DO and TA levels were experimentally manipulated and pH declined as a result of metabolic excretions, approaching levels found to cause growth reductions in chronic bioassays (Harris 1999). In Chapter 7, water exchange was interrupted for 60-90 minutes, and the changes in DO, ammonia and pH were due primarily to abalone metabolism on Day 1, and a combination of abalone metabolism and accumulated organic wastes at Day 5. The organic wastes accentuated the decline in pH and DO, but had little impact on TA, with TA also declining on Day 5 as well.

Should influent water flow be reduced for an extended period at commercial stocking densities, then a range of water quality factors will be altered, including declines in dissolved oxygen and pH and increases in environmental ammonia, which may well affect the health or growth of the stock. If organic wastes are not removed before or during any interruption or reduction in water exchange, these problems will be further compounded by factors such as the presence of sulphide and an increase in bacterial numbers.

The consequences of an interruption to water flow in the shallow plug-flow tanks popular in South Australia and Victoria will depend more on ambient temperature. While stress on the exposed animals is likely to be minimal as long as ambient air temperature remains within the lethal tolerance, and the abalone are kept moist, restoration of water flow in the shortest time is essential to prevent



major stock losses. Should an interruption or reduction in influent water flow occur in the deeper, aerated Taiwanese style tanks more common in Tasmania, the effect on the stock is less dependent on air temperatures, and the aquaculturist has several hours before restoration of water exchange becomes urgent.

This study also investigated water quality in different micro-environments found in abalone growout tanks, and their relationship with the bulk water quality. Chapter 7 (temporal effects) shows that the water quality within the accumulated sediment is acidic, oxygen depleted and ammonia enriched compared to the bulk water, yet analysis of water samples drawn from under the refuges in Chapter 5 (stocking density) revealed no significant difference from the bulk water in terms of pH, DO or TA during normal water exchange. The absence of any differences suggests that there is sufficient water exchange between these two environments to maintain comparable water quality. Such exchange may have been facilitated in this system by the use of aquarium pumps to maintain constant water current between treatments. While not examined in this study, it would be expected that accumulated organic wastes under the refuges would have a greater effect on water quality under the refuges should normal water exchange be interrupted.

Diet composition had no significant impact on the bulk water quality parameters measured. While the composition and pore-water within the wastes did change with composition, these changes were not reflected in bulk water quality readings.

#### **9.4 Nitrogen Budgets**

Abalone were shown to generate a similar environmental load in terms of nitrogen to other cultured species (Chapter 8 (budgets)). Since a substantial amount of the nitrogen leaving the abalone tanks was in the organic wastes, which is relatively easy to retain with settling ponds, it is not difficult to reduce the net load on receiving waters. Potentially the hardest component of abalone wastes to retain is the fine particulate material generated by the grazing action of the radula. More research is also required to investigate the “other losses” component of the nitrogen

models developed in Chapter 8, to determine how they can be also be reduced or eliminated. The soluble component, primarily ammonia, can be removed by the use of algal scrubbers, which has been successfully used in Israel in poly-culture systems. It is likely algae growing in the open drains present on most abalone farms will act in this manner.

## 9.5 Conclusions

Otherwise healthy blacklip and greenlip abalone were shown to be very tolerant of intermittent degradations in water quality, which is a consequence of their capacity to cope with functional hypoxia.

While very low oxygen levels (60%) seemed to offer some protection from chronic exposure to low levels of environmental ammonia, the same levels of ammonia combined with modest declines in dissolved oxygen (80%) had a greater impact on growth with some evidence that it was more deleterious than environmental ammonia or low dissolved oxygen levels as individual variables. These effects on growth could be related to a disruption in normal ion exchange and serum chemistry, which would be expected to reduce the amount of energy available for somatic growth.

An increase in stocking density reduced growth rates in greenlip abalone, which was alleviated by increasing the level of refuge provision at medium and high densities. This was related to the increase in area available for the quiescent resting period, and was probably due to a reduction in the energy required for locomotion during the foraging excursion allowing more energy to be utilized for growth.

Abalone metabolism and accumulated organic wastes have modest effects on bulk water quality provided adequate water exchange is maintained. If influent water flow is interrupted or reduced, both factors will contribute to declines in dissolved oxygen and pH, and increases in ammonia level.

Abalone were also shown to generate a similar nitrogen load to other species, and assimilate similar amounts of nitrogen. As with other species, retention of solid wastes will significantly reduce the nitrogen load on the environment.

Trial		Treatments	Effect on Growth			
			Blacklip		Greenlip	
			SGR-L	SGR-W	SGR-L	SGR-W
Low dissolved oxygen with elevated ammonia	intermittent exposure (Chapter 2)	0-8 exposures for 8 hours to over 8 weeks	no significant effects	no significant effects	no significant effects	no significant effects
Low dissolved oxygen with elevated ammonia	chronic exposure (Chapter 3)	Control	0.033	0.143	0.113	0.32
		MO/LA	0.033	0.086	0.107	0.274
		MO/MA	0.021	-0.027	0.06	0.085
		MO/HA	0.01	-0.048	0.043	0.164
		LO/LA	0.22	0.184	0.064	0.442
		LO/MA	0.014	0.114	0.081	0.427
Stocking density & refuge provision	Chapter 5	120/0			0.11	0.38
		120/2			0.10	0.34
		120/4			0.10	0.35
		240/0			0.07	0.26
		240/4			0.08	0.27
		240/8			0.09	0.29
		360/0			0.07	0.28
		360/6			0.08	0.24
		360/12			0.09	0.30

Table 9.1 Summary of growth data from the present study. Treatments for stocking density/refuge provision are given as numbers of abalone per tank/number of refuges per tank. Treatments for chronic exposure to low dissolved oxygen and elevated ammonia are given as oxygen level/ammonia concentration (MO=76-85, LO=56-64 % saturation; LA=1.06-1.44 mg.L<sup>-1</sup>, MA=1.88-2.48 mg.L<sup>-1</sup> & HA=8.07 mg.L<sup>-1</sup> TA).












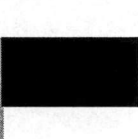

Data Source		Total Ammonia (mg.L <sup>-1</sup> )	Unionised Ammonia (µg.L <sup>-1</sup> )	% Oxygen Saturation	Oxygen Level (mg.L <sup>-1</sup> )	Significant Growth Reductions	
						Length	Weight
Harris <i>et al.</i> , 1997a		1.01	25				
		1.46	31				
		2.65	54				
		6.16	110				
		9.04	188				
Harris <i>et al.</i> , 1999a				100	7.7		
				81	6.2		
				73	5.6		
				63	4.9		
				55	4.2		
Chapter 2	MO/LA	1.06	24	83	6.19		
	MO/MA	1.88	40	76	5.58		
	MO/HA	8.07	197	85	6.27		
	LO/LA	1.44	38	64	4.89		
	LO/MA	2.48	59	56	4.29		

Table 9.2. Effects on growth of exposure to elevated environmental ammonia and low dissolved oxygen levels for greenlip abalone as individual exposures (either DO or ammonia) or as a combination of low DO with elevated ammonia. Solid blocks indicate growth rates significantly different from the control.

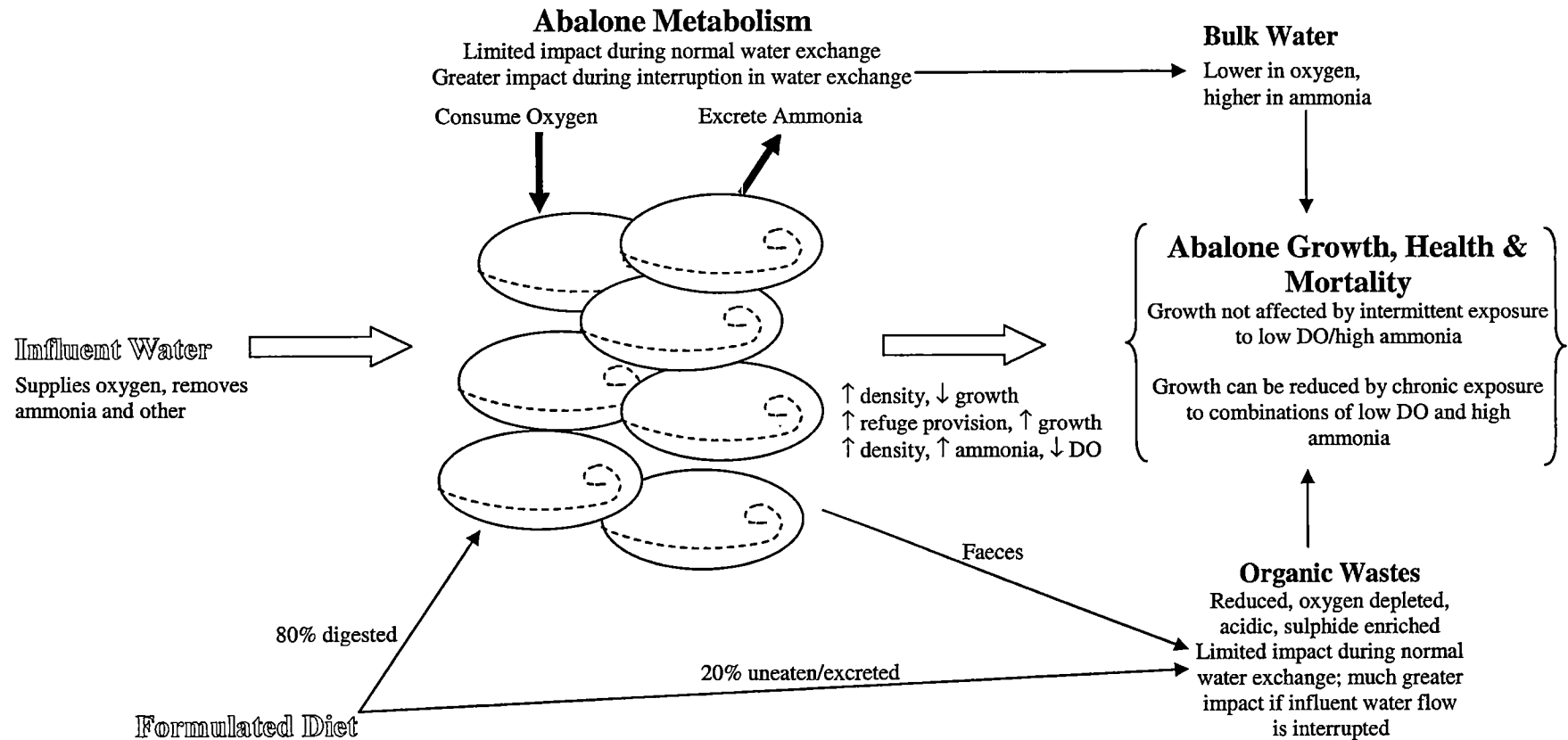


Figure 9.1 – Summary of the factors influencing growth and water quality in abalone systems covered in this study. More detail is given in Table 9.1 and text.

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